

The Distribution of Opiates, Cocaine and their
Metabolites in Skeletal Muscle Tissue and
Vitreous Humour as an Aid to Post-mortem
Toxicological Interpretation

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*This thesis is dedicated to my mother, Gaynor Bown-Moll, for her constant
enthusiasm and support*

Abstract

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Post-mortem blood drug concentrations vary greatly and as a consequence of post-mortem change and redistribution may not reflect the concentration at the time of death. Tissues that are located away from central drug reservoirs and that lack esterase activity, e.g. muscle and vitreous humour (VH), have the potential to provide more reliable post-mortem toxicological specimens. In the absence of a blood sample the toxicologist may have to rely on such tissues yet few studies have been undertaken to examine the relationship between drugs in blood and less conventional tissues at the time of death.

The purpose of this study was to investigate the distribution of opiates (heroin specific compounds) and cocaine and their respective metabolites in VH and muscle with a view to elucidating the interpretive value of these tissues. Analytical methods were developed and validated to measure drug concentrations in blood, VH and muscle, including throughout the *rectus femoris* thigh muscle, in cases of drug related death. To assist with interpretation of drug concentrations measured in post-mortem tissues the *in vitro* stability of cocaine and 6-acetylmorphine (6AM) was examined during the putrefactive process and under different storage conditions. Relationships between blood and tissue drug concentrations were assessed in relation to case circumstances with particular focus on the approximation of survival time.

In contrast to a report previously published in the literature, this study found the concentration of cocaine, and its metabolites, benzoylecgonine (BZE) and cocaethylene (COET), to be uniformly distributed throughout the thigh muscle ($n = 7$). Concentrations of cocaine in muscle were markedly higher than in blood and correlated well with the blood. The stability of cocaine in muscle tissue was found to greatly exceed that in blood and VH. These preliminary results also indicated that the cocaine to BZE ratio measured in both muscle and VH may be of value in the assessment of survival time. These findings promote the use of muscle as a toxicological specimen for cocaine determinations. Further work is required to validate these findings and to examine the distribution of opiates in muscle, which could not be assessed in this study. The relationship between femoral blood and vitreous humour morphine concentration ($n = 70$) was found to be dependent on survival time and possibly influenced by accumulation of morphine in the VH. These findings demonstrate that the concentration of morphine in blood cannot be inferred from that measured in the VH. The VH provided a useful adjunct to interpretation owing to the prolonged detection of 6AM in this matrix. The addition of 1.5% sodium fluoride to VH was found to be essential for 6AM stability during storage. The utility of rapidly metabolised heroin specific compounds in blood as indicators of survival period following heroin intake and the role of concomitant drug consumption in heroin fatalities was also discussed in this thesis.

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Declaration

I, the undersigned, hereby declare that this dissertation is the result of my own works and it includes nothing which is the outcome of works done in collaboration unless otherwise stated. This dissertation has not been submitted for any other degree or diploma.

Chapter 1.0 Introduction

The majority of drug related deaths in the UK relate to opiate use, chiefly heroin/morphine (Davies et al. 2010). The acute toxicity of heroin, especially after intravenous administration, is high compared with other drugs of abuse (Drummer 2005). There is considerable evidence that many instances of opioid overdose are due to the combined effects of opioids with other drugs (Monforte 1977; Darke et al. 1996; Zador et al. 1996; Platt 1997; McGregor et al. 1998; Ochoa et al. 2001; Fugelstad et al. 2002; Coffin et al. 2003; Darke et al. 2007) and polydrug use is highly prevalent among illicit drug users in the UK (Davies et al. 2010; Smith et al. 2011). Cocaine is reported in the toxicological analysis of a high proportion of drug related deaths, generally in combination with opiates and other substances (EMCDDA 2007). The use of heroin, cocaine and alcohol in any combination can significantly increase the risk of acute overdose (Monforte 1977; Platt 1997; Ochoa et al. 2001).

There are several challenges in the interpretation of post-mortem drug and metabolite concentrations. Post-mortem blood drug concentrations vary greatly depending on the dosage, route of administration, individual tolerance, age, state of health, concomitant use of other drugs, the period of survival after drug intake, and the manner of sample storage. Correlating a specific blood concentration to toxicity is difficult, even in a living individual (Ferner 2008). The situation after death is even more complex because the concentrations measured at autopsy do not necessarily reflect those at the time of death. It is known that weakly basic drugs, such as morphine and cocaine, may be subject to a process known as post-mortem redistribution, where drugs move from areas of high concentration in the central viscera to areas of lower concentration after death (Prouty and Anderson 1987; Pounder and Jones 1990; 1999). Residual metabolic enzyme activity, known to occur in the early post-mortem period, can contribute significantly to changes in drug concentration (Pelissier-Alicot et al. 2003), and represents a particular problem for cocaine (Logan et al. 1997). As time between death and sampling increases so does the likelihood that site- and time-dependent changes in blood drug concentrations will have occurred (Prouty and Anderson 1990).

Notwithstanding the post-mortem phenomena of site- and time- dependency in the interpretation of a drug concentration, blood has become the specimen of choice for ascertaining pharmacological effects in cases of drug-related death. However, blood is

not always available at autopsy, particularly when the body is beginning to decompose. In such instances, toxicologists must rely on the analysis of remaining tissues that are less affected by putrefaction, e.g. vitreous humour (VH) and skeletal muscle. Examples of such cases include exhumed bodies, cases of suicide where the body may not be found for days or even longer, and bodies recovered from remote sites in cases of murder and sexual assault. Few studies have been undertaken to examine the relationship between drugs in blood and less conventional tissues at the time of death.

Vitreous humour and skeletal muscle have potential advantages as toxicological specimens. They are located well away from drug reservoirs in the central body compartment and as a result are less likely to show changes in drug concentration attributable to redistribution in the early post-mortem period. These tissues also lack hydrolytic esterase activity and so drugs broken down via esterase mediated hydrolysis, such as cocaine and 6AM, should have increased stability compared with in blood. The principal utility of VH for cocaine and 6AM determinations is reported to be the extended window of detection it provides (Pragst et al. 1999; Antonides et al. 2007). Studies on the relationship between blood and vitreous humour, particularly for opiates, are relatively limited and often based on small sample numbers or individual cases. There is subsequently conflicting evidence as to whether a relationship exists between these matrices for cocaine and its metabolites (Logan and Stafford 1990; Mackey-Bojack et al. 2000; Duer et al. 2006) and for morphine (Gerostamoulos and Drummer 1997; Scott and Oliver 1999; Stephen et al. 2006). Drug concentration data in muscle tissue has barely been reported, yet it is known that the stability of cocaine is increased substantially in this tissue relative to blood (Moriya and Hashimoto 1996). The general lack of interest in muscle as a toxicological specimen may be attributed to the observation of great intra- and inter-muscular variation in the concentration of some basic drugs (Williams and Pounder 1997; Langford et al. 1998). Consequently the use of muscle tissue has been considered for qualitative rather than quantitative drug determination. The within muscle distribution of opiates and cocaine has not been reported.

Without further understanding of the relationship between drug concentrations in these less conventional tissue and blood, and how the relationship may be affected by the within tissue distribution, a reliable interpretation of an analytical result obtained in the

absence of a blood sample is impossible. Further, the changes in drug and metabolite concentrations that occur in these matrices over time must be well understood as this will directly affect the relationship with blood as a function of the post-mortem interval. Drug stability in alternative matrices, during the putrefactive process and during sample storage, represents another area in which published data is currently lacking.

1.1 Study aim and objectives

The purpose of this study was to investigate the distribution of opiates (specifically morphine and other heroin specific compounds), cocaine and their respective metabolites in vitreous fluid and muscle with a view to elucidating the interpretive value of these tissues in post-mortem toxicological assessments. Using data compiled from autopsy cases and spiked animal tissue, this study aimed to address a number of questions currently unanswered in the scientific literature, including:

- 1) Is there a relationship between the concentration of opiates and cocaine (and their metabolites) in blood, muscle and VH at the time of death?
- 2) How significant is the sampling location within a given tissue in terms of the measured drug concentration?
- 3) How do the concentrations of opiates and cocaine within these tissues change during the putrefactive process and during sample storage?

The objectives were:

- 1) To review relevant published scientific literature to assist in the interpretation of the toxicological findings (Chapter 2), including:
 - the pharmacokinetic properties of the investigated drugs
 - factors affecting post-mortem drug concentrations and their subsequent interpretation
 - the current utility of VH and muscle in toxicological determinations
 - the stability of opiates and cocaine in blood, VH and muscle during putrefaction and sample storage
- 2) To develop analytical procedures for the simultaneous extraction, confirmation and quantification of opiates, cocaine and their respective metabolites using solid phase extraction and GC-MS/MS with subsequent validation to ensure accuracy, selectivity and consistency (Chapter 3).

- 3) To examine the stability of drugs broken down via hydrolytic esterase activity (cocaine and 6AM) by monitoring parent drug and metabolite concentration changes in spiked animal muscle, VH and blood sampled at increasing time intervals during the putrefactive process as an aid to interpretation of distribution data. And, to examine the effect of storage temperature and preservation on drug stability in the various tissues with the aim of making recommendations for optimal storage conditions (Chapter 4 and 5).
- 4) To examine tissue distribution in multiple cases of opiate and cocaine related death (Chapter 6 and 7) by measuring drug and drug metabolite concentrations within blood, VH and muscle, including multiple measurements throughout a single thigh muscle (*rectus femoris*), and to assess the relationship between blood and muscle/VH by:
 - determining the correlation between analyte concentrations measured in these tissues
 - comparing tissue to blood concentration ratios in different cases
 - comparing drug to drug metabolite ratios in different tissues
- 5) To elucidate the interpretive value of muscle and VH by assessing the relationship between these tissues and blood with respect to case circumstances, with particular focus on survival time (Chapter 6, 7 and 8).

Chapter 2.0 Literature Review

2.1 Drug-induced deaths in the UK

The European Monitoring Centre for Drugs and Drug Addiction defines drug-related deaths as deaths caused directly by the consumption of one or more illegal drugs, i.e. an ‘overdose’. The majority of drug-related deaths in the UK relate to opioid use, chiefly heroin/morphine and methadone, the main heroin substitution treatment drug used in the UK (Davies et al. 2010). In 2009 there were 2,092 drug-related deaths reported in the UK, 58% of which involved heroin/morphine (n = 1,210) and 11% involving cocaine (n = 238) (Davies et al. 2010). Drug deaths are on the rise in the UK, with the figure reported for 2009 representing an increase of 81.6% since 1996 (n = 1,152) (Davies et al. 2010). Drug related fatalities increasingly involve more than one drug, with opiates frequently detected in combination with alcohol, benzodiazepines, particularly diazepam, and cocaine (Seymour et al. 2000; Oliver and Keen 2002; Davies et al. 2010). Drugs that were mentioned most frequently on death certificates in the UK between 2002 and 2009 are displayed in Table 1 (Davies et al. 2010).

Table 1 Drug mentions on death certificates in the United Kingdom, 2002 to 2009 (Davies et al. 2010)

	2002	2003	2004	2005	2006	2007	2008	2009
Heroin/morphine*	1,118	883	977	1,043	985	1,130	1,243	1,210
Methadone	300	292	300	292	339	441	565	582
Cocaine	161	161	192	221	224	246	325	238
Diazepam	356	282	217	205	186	223	489	300

*Morphine is the major metabolite of heroin and is the main drug detected at autopsy following heroin use.

Overdose is a particular risk among injecting drug users (Frischer et al. 1997; Hall and Darke 1998). Intravenous injection (IV) causes a rapid increase in brain levels of the drug increasing the likelihood of overdose via this route of administration (Frischer et al. 1997; Kaye and Darke 2004; Karch 2009;). Loss of drug tolerance is a further risk factor associated with drug overdose, particularly opioid overdose. Chronic users build up neuroadaptive tolerance to the drug and require progressively larger doses to attain the same effect. Following a period of reduced drug use, or abstinence, the

administration of a drug at previous high dosage can prove fatal (White and Irvine 1999).

Polydrug use has been documented as a key risk factor in overdose and overdose mortality (Monforte 1977; Goldberger et al. 1994; Darke and Zador 1996; Zador et al. 1996). The concomitant use of opiate with other central nervous system (CNS) depressants, particularly alcohol and benzodiazepines, has been repeatedly shown to increase the risk of opiates overdose (Monforte 1977; Darke et al. 1996; Zador et al. 1996; McGregor et al. 1998; Coffin et al. 2003; Darke et al. 2007). The combined use of opiates and cocaine has also been associated with a greater risk of overdose (Platt 1997; Ochoa et al. 2001). Combinations of narcotics and analgesics were the most frequently reported in a large study of medication-related emergency department visits (Cone et al. 2003).

2.2 Opiates (heroin and metabolites)

Heroin, or diacetylmorphine, is a semi-synthetic opioid drug synthesised by the coupling of two acetyl groups to the 3- and 6-hydroxyl groups of morphine. Morphine itself is a tropane alkaloid obtained from the latex of the opium poppy, *Papaver somniferum*, and is the primary constituent of crude opium. Morphine is prototypical of the narcotic analgesics and is the drug of choice for the treatment of chronic pain syndromes and myocardial infarction (Karch 2004). On entering the bloodstream heroin is rapidly hydrolysed back to morphine via the formation of the biologically active intermediate, 6-acetylmorphine (6AM) (Boerner et al. 1975; Inturrisi et al. 1984; Rentsch et al. 2001). Because heroin crosses the blood-brain barrier much more rapidly than morphine, heroin may be considered a pro-drug for the systemic delivery of morphine (Inturrisi et al. 1983).

The medicinal use of heroin has been banned in most countries due to its addictive properties but under the name diamorphine, heroin is a legally prescribed controlled drug in the UK (it is a Class A drug controlled by the Misuse of Drugs Act, 1971). The medicinal uses of diamorphine include treatment for acute, post-surgical and chronic pain. In the illicit use of heroin, the drug is self-administered as the hydrochloride salt, or base, typically by intramuscular or IV injection, by nasal insufflation, and by

smoking. The naive dose of a few milligrams escalates rapidly and the typical addict attending a clinic administers around 750 mg of heroin daily (Henry 1999).

2.2.1 Absorption

Heroin passes the blood-brain barrier rapidly resulting in an almost instant effect (Oldendor et al. 1972). Peak heroin concentrations in venous blood are achieved within 1 - 2 minutes after IV, intranasal (IN), intramuscular (IM) and smoked administration (Jenkins et al. 1994; Skopp et al. 1997). When heroin is administered intravenously or by the smoked route, conversion to 6AM and subsequently to morphine occurs almost instantaneously with peak concentrations of 6AM and morphine often reached around the same time as heroin (Jenkins et al. 1994; Skopp et al. 1997; Gyr et al. 2000). Following IV injection plasma half-lives ranging between 1.75 – 7.6 minutes for heroin and 9.3 – 49 minutes for 6AM have been reported (Jenkins et al. 1994; Gyr et al. 2000; Rentsch et al. 2001; Rook et al. 2006a). Morphine concentrations generally peak at 2 – 7.8 minutes and is detectable in plasma for much longer than heroin and 6AM with a reported half-life of 109 – 287 minutes (Jenkins et al. 1994; Gyr et al. 2000; Rentsch et al. 2001).

The absorption of heroin through the lungs occurs very rapidly and is virtually complete immediately following smoking (Rook et al. 2006a). The bioavailability of inhaled heroin is estimated to be approximately 52% (Rook et al. 2006a) although there may be much variation depending on the users experience and the method of volatilisation, i.e. heating on foil ('chasing the dragon') or smoking cigarettes laced with or dipped in heroin powder (Kramer et al. 1991; Strang et al. 1997a). In a large study on the pharmacokinetics of heroin in heroin dependent individuals (n = 106) Rook et al. (2006a) reported that peak plasma concentrations of heroin, 6AM and morphine were on average two to four times lower after inhalation than following IV administration. Despite the lower plasma concentrations reported after inhalation, the subjective effects following this route were rated more positively compared to injection of the drug. The mean residence time of heroin in the blood after the IV and smoked routes is reported to be less than ten minutes (Jenkins et al. 1994).

The pharmacokinetic profile of IN heroin is reported to be similar to that of the IM route although peak concentrations of heroin and 6AM were higher following the IM route

(Cone et al. 1993; Skopp et al. 1997). IM administered heroin is reported to result in sustained heroin exposures due to lack of esterase activity and therefore minimum deacetylation in the muscle. Cone et al. (1993) reported that the IN administration of 12 mg heroin HCl produced concomitant physiological and behavioural effects over a similar time course to those produced by administration of 6 mg IM heroin indicating a 2:1 relative potency of the IM route compared with the IN route. The bioavailability of IM administered heroin is reported to average 380% (Girardin et al. 2003) whereas the bioavailability for morphine administered by the same route is estimated to be 100% (Karch 2009).

Oral administration results in slow and inefficient delivery of the drug to the brain due to hydrolysis in the gastrointestinal tract and loss due to extensive first pass metabolism. As a result, heroin and 6AM are rarely detected in the blood following this route of administration (Gyr et al. 2000; Girardin et al. 2003). Morphine entry into the systemic circulation has been shown to be more rapid after oral heroin than after oral morphine (Halbsguth et al. 2008). The bioavailability of morphine following oral morphine is estimated to be approximately 33% (Lotsch et al. 1999) whereas morphine bioavailability in chronic opiate users following oral heroin was estimated to be 64 - 72% (Halbsguth et al. 2008). In the latter study, chronic users achieved peak morphine concentrations almost three-fold that measured in naive users and in approximately half the time. These authors attributed the increase in bioavailability and peak concentrations in chronic users to repeated opioid use decreasing the expression or function of morphine metabolising enzymes in the intestine.

Following the administration of sustained release oral morphine preparations, the time to reach peak plasma morphine concentration and the elimination half-life is increased in comparison with immediate release preparations and can vary greatly depending on the preparation (Baselt 2004). The literature clearly shows that the blood concentration, rate of elimination and duration of effect of heroin/morphine is dependent on the specific drug preparation, the route of administration as well as the frequency of drug use.

2.2.2 Metabolism

In vivo the 3-acetyl group of heroin is rapidly deacetylated ($T_{1/2} = 3.24 - 3.7$ minutes) (Rook et al. 2006a) forming 6AM which is then further hydrolysed at the 6-position, albeit at a slower rate ($T_{1/2} = 22 - 26$ minutes) (Rook et al. 2006a), forming morphine (Boerner et al. 1975; Inturrisi et al. 1984; Rentsch et al. 2001). In blood, hydrolysis of heroin to 6AM occurs spontaneously (Smith et al. 1978) and by hydrolytic esterase activity. Esterase mediated hydrolysis is catalysed by serum butyrylcholinesterase (BuChE) (EC 3.1.1.8) (Lockridge et al. 1980; Salmon et al. 1999) and at a slower rate by erythrocyte acetylcholinesterase (AChE) (EC 3.1.1.7) (Salmon et al. 1999). In the liver this reaction is catalysed by human carboxylesterase (hCE) (EC 3.1.1.1). Two types of hCE have been identified in human liver, hCE-1 and hCE-2 (Kamendulis et al. 1996; Pindel et al. 1997). The order of catalytic efficiency in the formation of 6AM is hCE-2 >> hCE-1 > BuChE > AChE (Kamendulis et al. 1996; Pindel et al. 1997; Salmon et al. 1999). AChE, but not BuChE, further catalyses the deacetylation of 6AM to form morphine (Salmon et al. 1999). In the hydrolysis of 6AM to morphine hCE-2 has considerably greater catalytic efficiency compared with hCE-1 (Kamendulis et al. 1996)

Satoh et al. (2002) reported the order of tissue specific expression for hCE-1 to be liver >> heart > stomach > kidneys = testis = spleen > colon > other tissues (including brain, lung and small intestine). For hCE-2 the order of expression was liver > small intestine > colon > heart. Both hCE-1 and -2 expression was absent in muscle tissue. Carboxylesterase (CE) has also been identified in human plasma (Welch et al. 1995) although it is most likely synthesized in liver and then secreted into the circulation via the Golgi apparatus (Satoh and Hosokawa 1998) and is not thought to have significant activity in human plasma (Li et al. 2005). Humans also express CE (not otherwise specified) in the macrophage (Munger et al. 1991) and monocytes (Saboori and Newcombe 1990) of white blood cells. Lymphocytes have been implicated in the conversion of 6AM to morphine within cell cultures although the mechanism was thought not to be enzyme mediated since specific and non-specific esterase inhibitors did not prevent hydrolysis (Hutchinson and Somogyi 2002).

Morphine itself undergoes metabolism via conjugation with glucuronic acid. Glucuronidation of the free phenolic hydroxyl groups at the 3- and 6- positions of morphine forms morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G)

respectively. These reactions, catalysed primarily by Uridine diphosphate-Glucuronosyltransferase-2B7 (UGT2B7) (Coffman et al. 1997), occur mainly in the liver and to a lesser extent in the kidneys, intestines (Brunk and Delle 1974) and brain (Wahlstrom et al. 1988), although morphine glucuronides do not appear to be formed to any significant degree in brain (Gerostamoulos and Drummer 1997). In general, M3G is the major metabolic product, formed at a ratio of approximately 7:1 (M3G:M6G) (Coffman et al. 1997). The glucuronides are not further metabolised but excreted via the kidney. Morphine also undergoes *N*-demethylation by hepatic cytochrome (CYP)-3A4 and to a lesser extent CYP2C8 to form the minor but active metabolite normorphine (Projean et al. 2003). Other morphine metabolites, such as morphine 3-ethereal sulphate, morphine 3,6-diglucuronide and normorphine 3-glucuronide are formed in very small amounts (Yeh et al. 1977; 1979). The plasma half-life of morphine is reported to be 1.3 – 6.7 hours (Baselt, 2004).

Codeine undergoes *O*-demethylation to morphine, a conversion catalysed by CYP2D6 (Lotsch et al. 2004; Kreek et al. 2005). Codeine is conjugated by UGT2B4 and UGT2B7 to form codeine-6-glucuronide (C6G) (Coffman et al. 1997). Because codeine has a methoxy group on the 3-position it is not converted to the 3-glucuronide. Small amounts of codeine may also be *N*-demethylated by CYP3A4 forming norcodeine (Yue and Sawe 1997), which is believed to be psychoactive (Fraser et al. 1960; Caraco et al. 1996). The half-life of codeine is reported to be 1.9 - 3.9 hours (Baselt, 2004). The metabolic pathway of heroin, morphine and codeine is displayed in Figure 1.

2.2.3 Tissue disposition

Heroin is rapidly distributed from the systemic circulation into tissues. Because the pKa of heroin (7.6) is close to physiological pH (pH 7.4), a large proportion is present in the lipophilic non-ionised form, thus favoring absorption (Klous et al. 2005). Heroin crosses the BBB within 15 – 20 seconds with approximately 68% of an IV dose absorbed into the brain (Moffat et al. 2004). 6AM is also highly lipophilic and crosses the BBB freely (Klous et al. 2005).

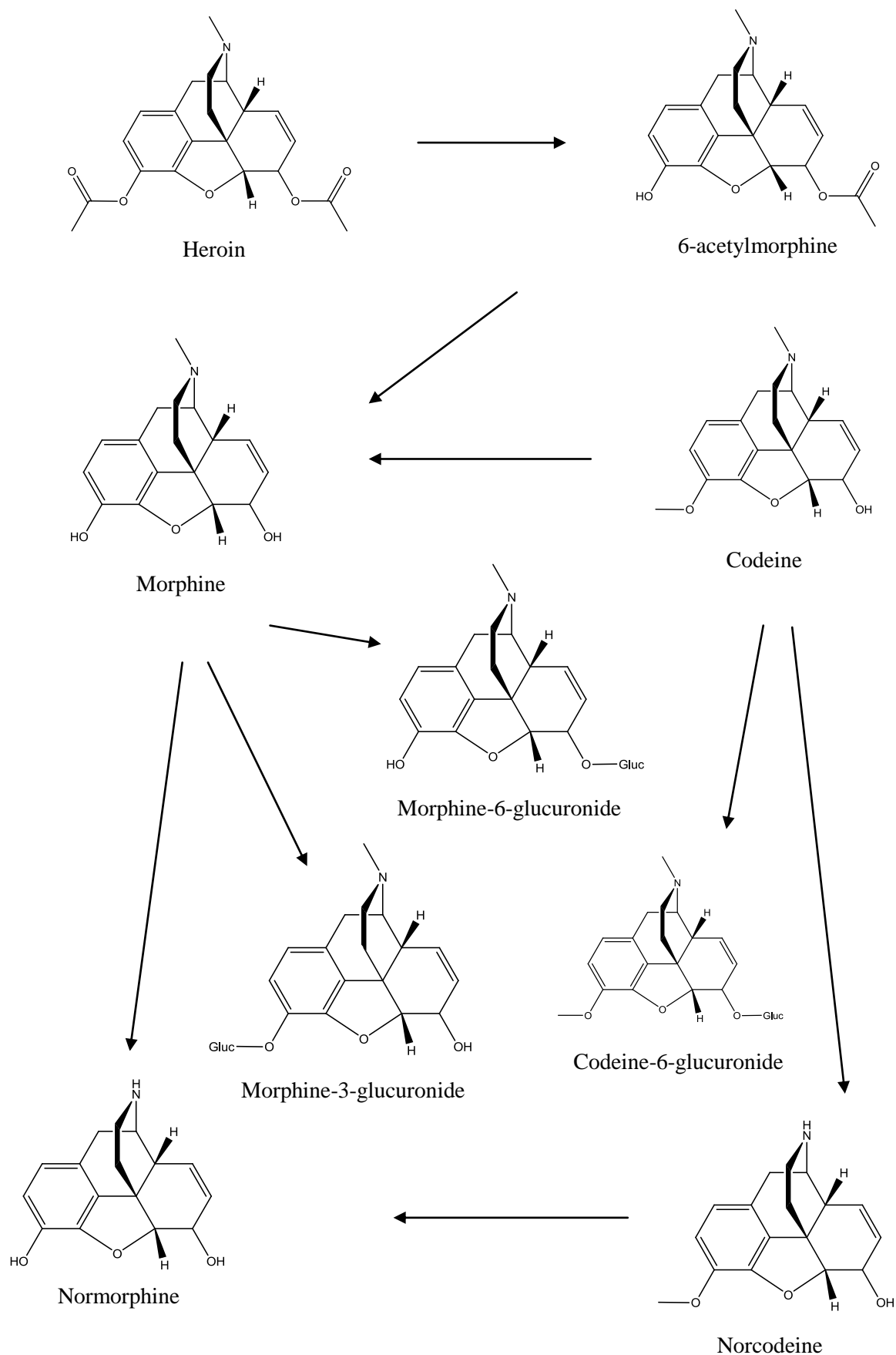


Figure 1 Metabolic pathway of heroin, morphine and codeine

Owing to its rapid metabolism heroin is rarely detected in biological fluids and tissues although its stability is reported to be increased in brain tissue (Karinen et al. 2009). 6AM is detected more frequently, particularly in tissues lacking esterase activity. 6AM concentrations in the brain and cerebrospinal fluid (CSF) have been found to be much higher than corresponding levels in the blood, liver, lung and kidney (Goldberger et al. 1994) (Table 2). Vitreous humour (VH) concentrations also typically exceed those measured in blood (Lin et al. 1997; Pragst et al. 1999; Wyman and Bultman 2004).

Owing to the relatively large volume of distribution (V_d) of morphine (3 – 5 L/kg) (Moffat et al. 2004), less than 2% of a given dose is to be found circulating in blood (Karch 2009). The V_d refers to the volume into which the total amount of a drug would have to be uniformly distributed to reach the concentration measured in plasma (Pelissier-Alicot et al. 2003). Drugs with a high V_d (> 1.0 L/kg) tend to be basic, lipophilic compounds which distribute into solid tissues. After IV administration morphine is rapidly distributed to the tissues receiving the highest blood flow, i.e. the lung, kidney, spleen and muscle, so that tissue concentrations generally reflect the relative blood flow (Brunk and Delle 1974). Morphine crosses the BBB at a much slower rate than heroin and 6AM (White and Irvine 1999). During life it is estimated that approximately half of the morphine circulating in plasma is protein bound (Osborne et al. 1990) although other estimates are more conservative (20 – 35%) (Moffat et al. 2004). Since the amount of morphine able to enter the brain is dependent on the amount of free morphine circulating in the plasma, concentrations in this matrix are typically low (Kintz et al. 1989; Goldberger et al. 1994) (Table 2). Morphine concentrations measured in the CSF are lower than in blood but appear to correlate well with the blood (Moriya and Hashimoto 1997). The concentrations of morphine in muscle have been found to be comparable to those in blood (Garriott 1991; Moriya and Hashimoto 1999; Drummer 2004). The accumulation of morphine in fat has been reported (Levisky et al. 2000). Both morphine and 6AM may also be detected in saliva (Jenkins et al. 1995), hair (Goldberger et al. 1991; Goldberger et al. 1994; Kintz and Mangin 1995) and in alternative matrices such as fingernails (Engelhart and Jenkins 2002), spinal cord, bone marrow and bone (Guillot et al. 2007). One of the most comprehensive studies on the tissue distribution of morphine was carried out by Kintz and co workers (1989). These authors measured free morphine concentrations in up to 16 human tissues and fluids. The data obtained by these authors in four cases of fatal heroin overdose are presented

in Table 2. In addition to the analysis of morphine, Goldberger et al. (1994) measured the concentration of 6AM in nine tissues in two individuals who died after taking heroin. This data is also displayed in Table 2.

M3G and M6G are highly polarized and minimally lipophilic (Gulaboski et al. 2007) and their ability to cross the BBB is significantly less than that of morphine (Wu et al. 1997). The V_d 's of M3G (0.14 L/kg) and M6G (0.15 L/kg) (Hunt et al. 1999) are so low that very little of either is found in tissues (Levisky et al. 2000; Karch 2009). M6G can be detected in brain, predominantly in the extracellular fluid (Stain-Textier et al. 1999), and its deposition in hair has also been reported (Rothe and Pragst 1995).

2.2.4 Elimination

The detection of heroin and its metabolites in biological specimens depends on the time between drug intake and specimen collection. Heroin itself is rarely detected in biological samples as a result of its very short half-life and also because significant deacetylation continues following specimen collection (Section 2.9.1) (Nakamura et al. 1975; Lockridge et al. 1980; Barrett et al. 1992; Romolo et al. 2003). After IV administration to heroin dependent patients, heroin was reported to be undetectable in plasma after 10 - 40 minutes (Rook et al. 2006a). The rate of clearance and elimination half-life of heroin does not appear to be dose dependent. In a study by Rentsch et al. (2001) high dose heroin (up to 210 mg) exhibited the same high clearance and short elimination half-life (2 - 5 minutes) as observed for low dose heroin. Heroin's primary metabolite, 6AM, also has poor stability and has been reported to be undetectable in plasma within 2 - 3 hours (Rook et al. 2006a). The window of detection for 6AM in urine is increased to between 2 and 8 hours after injection (Cone et al. 1991). Rentsch et al. (2001) reported that the clearance of 6AM considerably exceeded the usual hepatic and kidney blood flow, suggesting relevant contributions of additional tissues besides liver and kidney in the deacetylation of 6AM. Morphine and its glucuronides may be detected in urine for up to 72 hours after a single oral and intravenous morphine dose (Hasselstrom and Sawe 1993). The appearance of M3G in serum has been reported within 20 minutes of a morphine dose and exceeded that of morphine after 2 hours (Berkowitz 1976). The excretion half-life of total morphine following smoked and intravenous administration is reported to be 3.1 ± 0.30 hours with peak urinary concentrations observed at 2.3 – 9.3 hours (Smith et al. 2001).

Table 2 Tissue distribution of morphine (n = 6) and 6AM (n = 2) in six cases of fatal heroin overdose

Tissue	Free morphine (mg/L; mg/kg)						Mean	SD	6AM (mg/L; mg/kg)	
	Case #1	Case #2	Case #3	Case #4	Case #5	Case #6			Case #5	Case #6
Blood	0.021	0.801	0.173	0.057	0.208	0.082	0.22	0.29	0.011	0.016
Urine (TM)	0.207	6.69	0.832	6.55	0.218	0.655	2.5	3.2	0.018	0.848
Bile (TM)	0.087	1.253	0.363	0.248	NA	NA	0.49	0.52	NA	NA
Lung	NA	0.147	0.094	0.184	0.423	0.285	0.23	0.13	0.006	0.004
Liver	0.067	1.424	0.225	0.286	0.216	0.091	0.38	0.52	ND	Trace
Kidney	0.011	1.065	0.069	1.179	0.482	0.359	0.53	0.49	0.002	Trace
Heart	NA	0.102	0.044	0.087	NA	NA	0.078	0.030	NA	NA
Spleen	NA	0.95	0.106	NA	0.448	0.245	0.44	0.37	0.205	0.125
Brain	0.005	0.101	0.023	0.089	0.11	0.089	0.070	0.044	0.158	0.054
CSF	NA	NA	NA	NA	0.04	0.037	0.039	0.002	0.058	0.039
VH	0.353	NA	0.03	NA	NA	NA	0.19	0.23	NA	NA
Testicle	NA	NA	0.029	0.091	NA	NA	0.060	0.044	NA	NA
Muscle (psoas)	0.01	NA	0.038	NA	NA	NA	0.024	0.020	NA	NA
Tongue	NA	NA	0.049	NA	NA	NA	0.049		NA	NA
Hair	ND	NA	ND	0.008	ND	0.00007	0.002	0.004	Trace	0.0001
pancreas	NA	NA	0.145	NA	NA	NA	0.15		NA	NA

Case 1 – 4: adapted from Kintz et al. (1989); Case 5 – 6: adapted from Goldberger et al. (1994)

SD: standard deviation

Approximately 80% of a heroin dose is excreted in the 24 hour urine, predominantly as conjugated morphine, with 5 – 7% as free morphine, 1% as 6AM, 0.1% as unchanged drug, and trace amounts of other metabolites. In chronic users, faecal excretion may account for between 7 and 10% of a given dose (Hanks et al. 1987). Morphine is subject to a process known as enterohepatic recycling (Hasselstrom and Sawe 1993; Kilpatrick and Smith 2005; Villesen et al. 2006), whereby morphine glucuronides are deconjugated by the colonic flora following excretion via the bile with subsequent re-absorption of free morphine (Hasselstrom and Sawe 1993). In a study on the pharmacokinetics of morphine in seven healthy individuals, an average 20.8% of the morphine dose (5 mg) was unaccounted for and was attributed to enterohepatic cycling (Hasselstrom and Sawe 1993). The urinary excretion of morphine appears, to some extent, to be pH dependent, with the excretion of free morphine rising as the urine becomes more acidic and the excretion of the conjugated morphine rising with increasing alkalinity (Moffat et al. 2004).

The relative proportion of morphine and its metabolites present in biological samples is dependent on the route of administration. Because orally administered morphine undergoes extensive first pass metabolism relatively more normorphine and M6G are formed as compared with intravenous administration (Brunk and Delle 1974; Osborne et al. 1990). Relatively higher amounts of M6G have also been reported following heroin inhalation (Rook et al. 2006a). Owing to delayed absorption, the rate of morphine clearance after oral morphine administration is reportedly slower than after IV administration (Osborne et al. 1990). The same is not observed for oral and IV heroin, where morphine clearance is essentially the same (Halbsguth et al. 2008). Intranasal and IM administration of heroin is reported to produce similar metabolite excretion profiles (Cone et al. 1996).

Chronic exposure to street heroin has been shown to cause a relative increase in concentration of M6G. Chronic IV heroin users when administered either heroin or morphine showed a reduction in blood and urine M3G concentrations in favour of M6G with a significantly higher M6G/M3G ratio observed compared with non-heroin abusers (Antonilli et al. 2003). In healthy opiate naive subjects given IV morphine, the half-life of M3G (3.9 hours) was longer than that of M6G (2.6 hours) (Osborne et al. 1990). In a tolerant individual given IV heroin the half-life of M6G (11 hours) was much longer

than that of M3G (6 hours) (Gyr et al. 2000). The findings of these two studies demonstrate an overall increase in the half-lives of the glucuronides in regular opiate users as compared with naive users. The ability to excrete morphine glucuronides is impaired in individuals with renal disease or renal insufficiency (Spiehler and Brown 1987). Sawe et al. (1986) reported that the elimination half-life of M3G was on average 4 hours in normal volunteers and 50 hours in 11 uremic patients and thus the relative amount of metabolite to morphine might be much higher in individuals with renal conditions. Although morphine elimination is not reported to be altered by renal failure (Karch 2009) its rate may be dependent on an individual's glucuronidating ability, which can vary as a result of genetic polymorphisms (Karch 2009). Racial and inter-ethnic differences is a further consideration; Chinese subjects have been found to have a higher clearance rate for morphine than Caucasians, primarily because they form more glucuronide and at a faster rate than Caucasians (Zhou et al. 1993).

2.2.5 Pharmacology

Opioids produce their analgesic effects through activity at three major receptor subtypes: mu (μ), kappa (κ) and delta (δ). Morphine and most other clinically significant opiates produce their effects primarily at μ receptors which are widely distributed throughout the CNS and also in the gastrointestinal tract (Robert 2004). Of all of the opioid agonists, μ receptor agonists' display the strongest analgesic action and have the highest abuse liability (Kieffer 1999). Effects of μ receptor stimulation include euphoria, miosis (pupillary constriction) and a reduction in the responsiveness of respiratory centres to carbon dioxide inducing respiratory depression (Karch 2004). Stimulation of μ receptors in the chemoreceptor vomiting trigger zone of the medulla produce nausea and vomiting, both commonly observed side effects with opiate use (Karch 2004). Morphine also has pronounced effects on the gastrointestinal tract that results in a reduction in the peristaltic activity of the intestines leading to constipation (Karch 2004).

Heroin is two to three times more potent than morphine (Moffat et al. 2004), although it exhibits relatively low affinity for μ receptors (Inturrisi et al. 1983). Binding to μ receptors requires a free phenolic hydroxyl group in the morphinian structure (3-OH) which heroin does not possess. Thus, heroin is considered to be a pro-drug with its prolonged effects mediated by its more stable agonistic metabolites, 6AM and morphine

(Schulz and Goldstein 1973; Inturrisi et al. 1983). 6AM has been found to be more potent at the μ receptor than morphine (Way et al. 1960; Inturrisi et al. 1983; Selley et al. 2001). There is some evidence to suggest that heroin may exert some action at μ opioid receptor subtypes (Schuller et al. 1999) and that both heroin and 6AM exert action at the δ opioid receptors in the brain and spinal cord (Rady et al. 1994; Rady et al. 1997). The rapid delivery of heroin to the brain and almost instantaneous conversion to its active metabolites produces an intense rush when the drug is either smoked or injected intravenously (Robert 2004).

The relatively large number of active metabolites of heroin means that there is considerable potential for inter-individual and possibly intra-individual differences in drug effect due to differences in rate of metabolism (White and Irvine 1999). Esterases responsible for the conversion of heroin to 6AM and then to morphine are subject to genetic variation (Lockridge et al. 1980) and thus there are likely to be slow and rapid converters of heroin (White and Irvine 1999). Further, polymorphisms of the genes encoding for UDPGT enzymes have been described (Levesque et al. 1997) and these may be responsible for inherited variations in the glucuronidation of morphine and may contribute to the intra- and inter-subject variation in response to heroin administration.

Morphine's major metabolite, M3G, has virtually no affinity for the opioid receptor. M6G on the other hand is an agonist at μ and δ receptors and has been shown to possess significant biological activity (Osborne et al. 1989; Paul et al. 1989; Abbott and Franklin 1991). M6G has been shown to have greater analgesic potency than morphine in humans (Osborne et al. 1990; Osborne et al. 1992). This observation has been attributed to elevated concentrations of M6G relative to morphine in brain extracellular fluid (Paul et al. 1989; Stain et al. 1995). Morphine diffuses extensively within the brain cells whereas M6G is located almost exclusively outside of the brain cells, remaining available to bind at opioid receptors (Stain-Textier et al. 1999). Clinical evidence suggests that at least some if not most of the effects of morphine may be mediated by M6G, particularly in situations where the metabolite accumulates such as after chronic oral dosing (Sawe 1986) and in patients with renal failure (Osborne et al. 1993). A relative increase in potency after repeated oral dosing compared with after repeated IV doses has been observed (Hanks et al. 1987). There is also evidence that M6G has peripheral analgesic effects (Tegeder et al. 2003). After short-term morphine

administration, the contribution of M6G to both analgesia and central nervous effects is thought to be negligible (Lotsch 2005). Other minor morphine metabolites, such as morphine 3-etheral sulphate, morphine 3,6-diglucuronide and normorphine 3-glucuronide appear to be devoid of physiological effects (Yeh et al. 1977; 1979).

2.2.6 Toxicity

The typical triad of opioid intoxication consists of coma, pinpoint pupils and respiratory depression (Lehmann 1997). Additional toxic effects of morphine include apathy, cold and clammy skin, confusion, constipation, dizziness, drowsiness, hypotension, hypothermia, nausea, urinary retention and vomiting (Baselt, 2004). In healthy volunteers, impairment of cognition and motor control was observable at plasma morphine concentrations at or above 0.040 mg/L (Kerr et al. 1991). While small doses of morphine merely depress the respiratory rate, large doses cause respiratory arrest. Profound respiratory depression and the need for assisted ventilation corresponded with peak plasma concentrations of 0.8 – 2.6 mg/L following the intravenous infusion of 55 - 66 mg of morphine in surgical patients (Stanski et al. 1976). Suppression of respiratory drive accounts for the mechanism of death in most instances of opiate overdose (Karch 2009).

Non-fatal heroin overdose is common in injecting users. A British study found that 58% of 212 injecting users had overdosed at some stage in their life with 30% having experienced an overdose in the previous year (Bennett and Higgins 1999). Pulmonary conditions appear to be the most common complications of overdose, of which the most widely reported is oedema (Duberstein and Kaufman 1971; Dettmeyer et al. 2000; Warner-Smith et al. 2001). Heroin users are at significantly increased risk of hepatic disease and this is a common finding at autopsy (Passarino et al. 2005). Intravenous drug use is the most significant risk factor for hepatitis C (Warner-Smith et al. 2001). Cardiac complications associated with overdose have been reviewed by Ghurna and Nolan (2000) and include arrhythmia (Neaderthal and Calabro 1975), acute cardiomyopathy (Paranthaman and Khan 1976) and haemoglobinaemia (Smith and Glauser 1975). Meine et al. (2005) reported that in patients with acute coronary syndrome, the use of morphine, either alone, or in combination with nitro-glycerine, was associated with an increased mortality rate. Heroin overdose also has the potential to cause significant neurological damage through prolonged hypoxia although the drug

itself is not directly neurotoxic (Warner-Smith et al. 2001). With the exception of liver disease, systemic dysfunctions as a result of heroin/morphine abuse are not well defined because few studies compare the frequency of their occurrence in long-term heroin/morphine users with that observed in a non-drug using population (reviewed by Karch 2009).

There is considerable evidence that many instances of opioid overdose are due to the combined effects of opioids with other drugs. The major drugs implicated in this way are alcohol and benzodiazepines (Monforte 1977; Darke et al. 1996; Zador et al. 1996; McGregor et al. 1998; Coffin et al. 2003; Darke et al. 2007). These drugs by themselves are relatively weak respiratory depressants but when combined with a potent respiratory depressant, such as morphine, can augment the opioid effect on respiration (Darke et al. 1996; White and Irvine 1999). The combined use/injection of heroin and cocaine, or 'speedballing', is a long-established and popular practice used to obtain the rush from cocaine combined with the mellowing effect of heroin. Speedball injectors are reported to be 2.6 times more likely to overdose than those who inject heroin or cocaine alone. Evidence of a pharmacodynamic interaction of cocaine with heroin in cause of death has been reported (Polettini et al. 2005). The adulteration of heroin with illicit fentanyl, a highly lipophilic μ receptor agonist, has become increasingly common in the United States (Karch 2009). On a weight for weight bases fentanyl is 50 – 100 times more potent than morphine and may substantially increase the risk of overdose (Karch 2009).

2.3 Cocaine

Cocaine is one of the most potent of the naturally occurring central nervous system stimulants. It is a tropane alkaloid obtained from the plant *Erythroxylon coca* L., principally in Peru and Bolivia, and to a lesser extent in *Erythroxylon noveogranatense* H., of Columbia (Karch 2009). The cocaine alkaloid is found in the leaves of the plant and makes up approximately 0.5 – 1.0% of the plant material by weight (Aynilian et al. 1974). In the UK cocaine is a Class A drug controlled by the Misuse of Drugs Act, 1971 although its medical use as an anesthetic is permitted. Cocaine is rapidly absorbed after topical administration and has a vasoconstrictor action, thus enhancing its effectiveness as a local anaesthetic. The drug has been widely utilized for topical anaesthesia in ophthalmological and otorhinolaryngological procedures although the drug is rarely

used in this capacity today as other local anaesthetics are equally effective and much safer (Dollery 1997).

Owing to its stimulant properties, the illicit use of cocaine is widespread in many social, cultural and personal settings. In the hydrochloride salt form, the drug is administered nasally by insufflation, by IV injection, orally and by genital application. When taken by the inhaled route, cocaine is more commonly administered as the base form of the drug ('freebase' or the lower purity 'crack' cocaine) (Karch 2007). Large inter-subject variations occur in the absorption, distribution and elimination rates of cocaine (Javaid et al. 1983).

2.3.1 Absorption

The rate at which cocaine enters the central nervous system (CNS) is dependent upon blood concentration, which in turn is dependent upon the route of administration (Cone 1995). Owing to efficient absorption from lung alveoli, cocaine administration by the smoked route rapidly produces high peak concentrations. Similarly, IV injection delivers the drug directly to the circulation with high peak concentrations also achieved rapidly. Similar peak concentrations have been reported following administration of cocaine via both routes. After intravenous injection of 25 mg cocaine HCl and 42 mg administered by the smoking route, Cone (1995) reported average peak plasma concentrations of 0.230 and 0.227 mg/L respectively within 5 minutes. Other investigators have also reported nearly equal peak concentrations within five minutes after similar doses via the two routes of administration (Jeffcoat et al. 1989; Isenschmid et al. 1992). Peak subjective effects following intravenous injection and smoking are achieved at around 3 and 1.4 minutes respectively and these effects are short lived (Volkow et al. 2000). Smoking results in the most rapid delivery of cocaine to the brain (Javaid et al. 1983; Volkow et al. 2000) although its bioavailability after smoking depends on several factors including the temperature of volatilisation, the matrix upon which the cocaine is heated, and drug loss in main- and side-stream smoke (Martin et al. 1989). Cone (1995) reported an average bioavailability of approximately 70% when cocaine was smoked in glass pipes. The range over five subjects was 20 - 110% indicating that the individuals experience in using the smoking apparatus is also a major factor in determining the bioavailability of smoked cocaine.

Compared to the intravenous and smoked routes, and owing to slower absorption rates, intranasal and oral administration produces lower concentrations over a prolonged period. Following intranasal administration of doses ranging from 32 – 140 mg, peak plasma concentrations of 0.045 - 0.220 mg/L were reached within 30 - 66 minutes (Van Dyke et al. 1978; Wilkinson et al. 1980; Jeffcoat et al. 1989; Cone 1995). Estimates of bioavailability reported in the literature range from 25% (Wilkinson et al. 1980) to 94% (Cone 1995). Javaid et al. (1983) reported that intranasal bioavailability was dose-dependent with higher doses leading to increased absorption efficiency. The fraction absorbed following 64 and 96 mg doses was approximately 30% and 58% respectively. Following intranasal doses of 48 and 96 mg, peak subjective effects were achieved in 14.6 minutes with physiologic and psychotropic effects sustained for approximately 40 - 60 minutes thereafter (Volkow et al. 2000).

Orally administered cocaine takes approximately 30 minutes to enter the bloodstream and typically only a third of an oral dose is absorbed (Volkow et al. 2000). Holmstedt et al. (1979) reported peak plasma concentrations of 0.011 - 0.149 mg/L within 25 - 120 minutes following chewing of powdered coca leaves containing 17 – 48 mg of cocaine. Following oral administration of 140mg cocaine HCl an average peak plasma concentration of 0.210 mg/L was achieved at 60 minutes (Van Dyke et al. 1978). Peak subjective effects are achieved approximately 60 minutes following oral administration (Rush et al. 1999). While the onset of physiologic and psychotropic effects are slow following oral administration, the effects are sustained for approximately 60 minutes after their peak is attained (Van Dyke et al. 1978).

2.3.2 Metabolism

Cocaine is rapidly metabolised *in vivo* (Figure 2) forming two pharmacologically inactive metabolites, benzoylecgonine (BZE) and ecgonine methyl ester (EME). At physiological pH (7.4) cocaine undergoes spontaneous chemical hydrolysis resulting in loss of the methyl function to form BZE (Taylor et al. 1976; Baselt 1983; Inaba 1989; Isenschmid et al. 1989). In the blood the benzoyl ester group of cocaine is hydrolysed by plasma BuChE forming EME (Stewart et al. 1977). In blood both BZE and EME are further hydrolysed to form ecgonine (ECG) (Stewart et al. 1977; Skopp et al. 2001a). It has been proposed that BZE, which retains the benzoyl ester function of cocaine, undergoes enzyme mediated hydrolysis in its conversion to ECG and since EME retains

the methyl ester function of cocaine, this metabolite would undergo chemical hydrolysis to form ECG (Stewart et al. 1977; Skopp et al. 2001a). Another esterase responsible for the hydrolysis of cocaine and resulting in the formation of both BZE and EME is carboxylesterase (Dean et al. 1991). Specifically, hCE-1 catalyses the formation of BZE by hydrolysis of the methyl ester linkage and hCE-2 hydrolyses the benzoyl ester linkage to form EME (Dean et al. 1991; Pindel et al. 1997). Since hCE-1 and hCE-2 are highly expressed in the liver (Sato et al. 2002), both esterases are important for systemic clearance of cocaine from the blood through the liver. hCE-1 is more important for renal clearance with high expression in the kidney and hCE-2 has importance in the clearance of orally administered cocaine with high expression in the intestines and colon.

Cocaine also undergoes oxidative metabolism to form the minor but active metabolite, norcocaine. Hepatic CYP-450 isoforms of the 3A family are responsible for the majority of norcocaine production in humans (Leduc et al. 1993). This metabolite is further *N*-hydroxylated to the very toxic *N*-hydroxynorcocaine, a conversion catalysed by several members of the CYP family (1A, 2A, 3A, and possibly 3B) (Pellinen et al. 2000). Other minor metabolites of cocaine include *m*-hydroxy-BZE, *p*-hydroxy-BZE, *m*-hydroxy-cocaine and *p*-hydroxy-cocaine (Karch 2009). The main pyrolysis product formed following inhalation of crack cocaine is methyl ecgonidine which is further hydrolysed *in vivo* to produce ecgonidine (Toennes et al. 1999; Shimomura et al. 2001; Cardona et al. 2006). Both compounds serve as markers for this route of cocaine administration.

In the presence of ethanol, cocaine undergoes transesterification to form the pharmacologically active metabolite, cocaethylene (COET). This reaction is catalysed by the same liver esterase that converts cocaine to BZE, hCE-1. The reaction occurs preferentially to the normal hydrolysis reaction forming BZE (Dean et al. 1991; Boyer and Petersen 1992; Brzezinski et al. 1994; Bourland et al. 1998). Due to their structural similarity the metabolic pathways of cocaine and COET are very similar (Figure 2). COET is bio-transformed to form norcocaethylene and hydrolysed to form ecgonine ethyl ester (EEE).

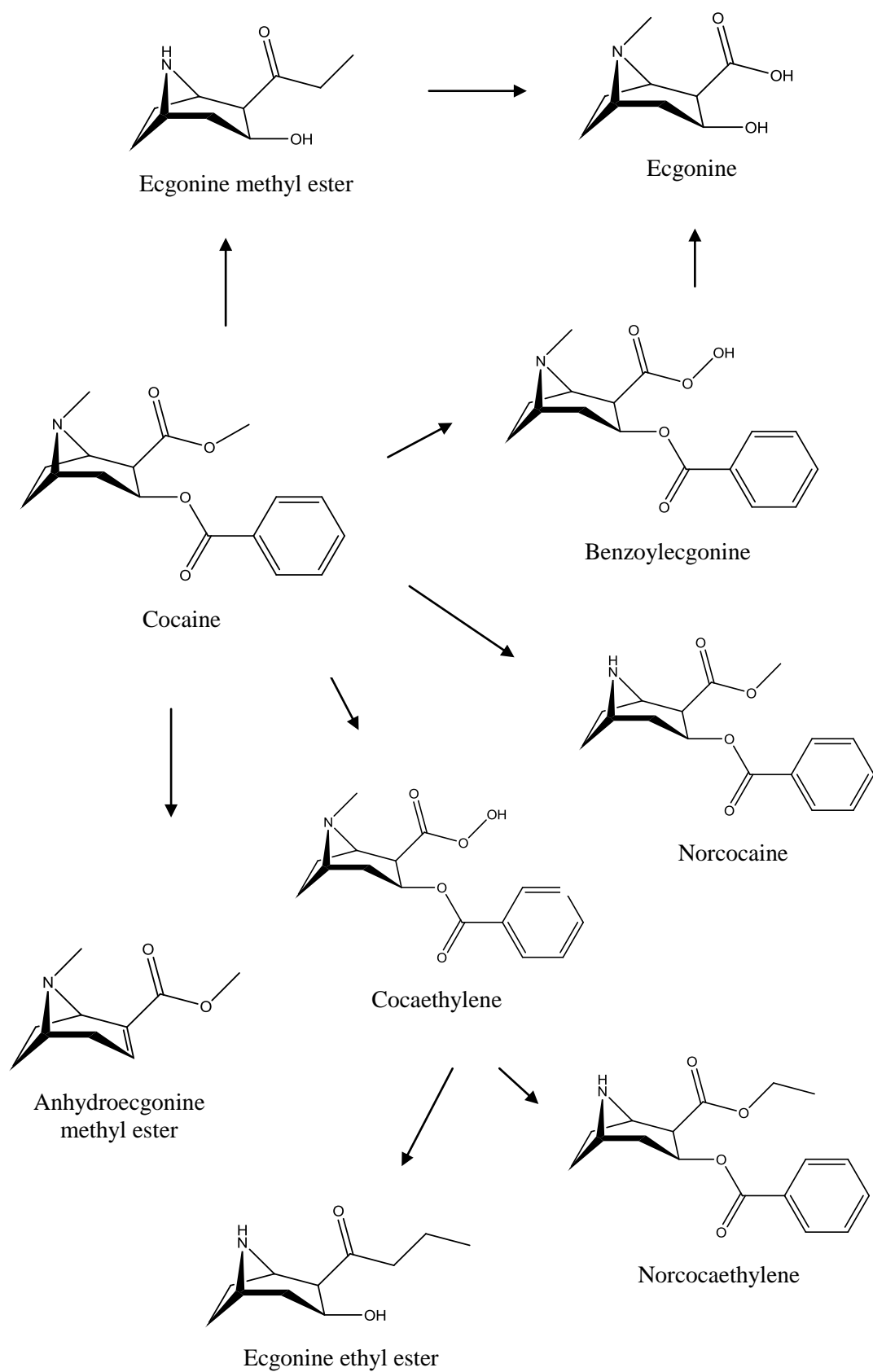


Figure 2 Metabolic pathway of cocaine

2.3.3 Tissue disposition

The high lipid solubility and large steady state V_d of cocaine (1.9 - 2.4 L/kg) (Hart et al. 2000) favours its distribution into tissues, particularly those with high lipid content such as the brain (Pelissier-Alicot et al. 2003). Following IV injection and smoking, cocaine is rapidly distributed to the central nervous system resulting in brain concentrations that are several-fold higher than concurrent blood concentrations (Misra et al. 1976; Spiehler and Reed 1985). Both cocaine and COET cross the BBB freely (Misra et al. 1975; Hearn et al. 1991a).

Following an IV dose of radio-labelled cocaine given to rats, the highest cocaine concentrations were found in the brain, spleen, kidney and lung (Busto et al. 1980). The lowest concentrations were found in the blood, heart and muscle. In rabbits given cocaine and ethanol orally, Moriya and Hashimoto (1996) found the concentrations of cocaine and COET to be of the order: brain > liver > muscle > blood. Nayak et al. (1976) compared cocaine disposition in acutely and chronically treated rats and found consistently higher levels of cocaine in the brain, fat and other tissues in the chronically treated group. Cocaine was detectable in the fat from chronically treated animals for up to four weeks following the final dose.

The concentration of COET in post-mortem blood and tissues is generally very modest, probably because the amount of alcohol present is the rate limiting step in COET production (Karch 2009). BZE is hydrophilic and does not cross the BBB (Misra et al. 1975; Rowbotham et al. 1990). The mean V_d of BZE is reported to be 0.7 L/kg (Ambre et al. 1991) and it is highly ionised at physiological pH ranges ($pK_a = 8.1$) so the metabolite should not readily distribute from the systemic circulation into surrounding tissues. However, prolonged excretion of both cocaine and BZE has been observed in abstinent cocaine abusers suggesting that both compounds are accumulated in deep body compartments (Cone and Weddington 1989; Burke et al. 1990).

Cocaine is also distributed from the blood circulation into the VH (Logan and Stafford 1990; McKinney et al. 1995; Mackey-Bojack et al. 2000; Duer et al. 2006). An apparent delay in the distribution into the VH has been reported with a subsequent delay in peak concentration (Antonides et al. 2007). Cocaine is incorporated in hair and in contrast to blood, where metabolite concentrations typically exceed that of the cocaine, the parent

drug is the predominant analyte detected in hair (Clauwaert et al. 2000; Kintz and Samyn 2002). Cocaine is also the predominant species detected in sweat and oral fluid (Kintz and Samyn 2002; Samyn et al. 2002). Cocaine disposition has also been described in alternative testing matrices such as meconium (Browne et al. 1992; Abusada et al. 1993), skin (Joseph et al. 1998; Yang et al. 2006) and fingernails (Roper-Miller et al. 2000; Engelhart and Jenkins 2002).

One of the most extensive studies on the tissue distribution of cocaine was conducted by Poklis et al. (1987), who measured the concentration of cocaine in 12 human tissues and fluids in five individuals who died after taking cocaine, two had smoked the drug and three had injected intravenously. The data obtained from these cases is presented in Table 3. This data demonstrates the wide variation between tissues in the concentration of cocaine, with the concentration measured being highly dependent on the time between dosing and death, individual variations in the rate of cocaine biotransformation, the level of esterase activity in the specific tissue and the extent of tissue accumulation as a result of dosing patterns.

2.3.4 Elimination

Cocaine is rapidly cleared from the bloodstream at an estimated rate of 2 L/min (Inaba et al. 1978). In the first 24 hours following cocaine administration, approximately 85 – 90 % of the dose is recovered in the urine. Depending on urine pH, 1 - 9% is eliminated as unchanged drug, 35 - 54% as BZE, 32 - 49 % as EME, and ecgonine (not quantified) (Fish and Wilson 1969; Inaba et al. 1978). Based on urinary excretion, several studies have reported a rapid elimination half-life when 0.3 - 3 mg/kg cocaine is administered intravenously (37 - 89 minutes), by the smoked route (38 - 82 minutes), and longer when intranasally administered (42 - 207 minutes) (Barnett et al. 1981; Javaid et al. 1983; Ambre 1985; Chow et al. 1985; Cone et al. 1988; Jeffcoat et al. 1989; Isenschmid et al. 1992; Cone 1995). Cone (1995) reported the appearance of BZE in plasma 15 - 30 minutes following cocaine administration with peak concentrations reached in 1.1 – 2.6 hours for the IV and smoked routes and at 2.9 – 3.8 hours for the intranasal route. EME could only be detected in the plasma of two subjects in this study and the concentration did not exceed 0.008 mg/L. Circulating EME is rapidly converted to ECG generally resulting in low EME concentrations in blood and plasma (Karch 2009).

Table 3 Tissue distribution of cocaine in five individuals who died after taking cocaine (mg/L or mg/kg)

	Case #1	Case #2	Case #3	Case #4	Case #5	Mean	SD
Route of administration	IV	S	IV	S	IV		
Blood	1.8	6.9	31	13	3.9	11	12
Bile	10	18		25	8.2	15	7.7
Brain	4	24	59	83	6.4	35	35
Heart	6.1	-	-	-	5.3	5.7	0.57
Kidney	26	26	58	53	34	39	15
Liver	1.6	17	6.5	10	15	10	6.3
Lung	3.4	-	69	24	27	31	28
Spleen	22	25	42	-	15	26	12
Skeletal muscle	6.1	-	48	30	-	28	21
Adipose tissue	1	-	5.8	-	0.7	2.5	2.9
Urine	39	41	270	-	27	94	117
Vitreous	2.4	-	-	14	-	8.2	8.2

Adapted from Poklis et al. (1987)

SD: Standard deviation; IV: Intravenous injection; S: smoked

Based on urinary excretion, elimination half lives of 4.5 - 4.7 and 3.1 hours have been reported for BZE and EME respectively (Ambre et al. 1988; Ambre et al. 1991).

When co-administered with ethanol, around 17% of a cocaine dose is converted to COET (Harris et al. 2003). On average the elimination half-life of COET is 1.7 hours and in the urine accounts for 0.7% of a cocaine dose in the first 24 hours (De La Torre et al. 1991; Jatlow et al. 1991; Perez-reyes et al. 1994). Because COET has lower affinity for hCE-1 compared to cocaine, it is detectable for much longer than cocaine, both in urine and blood (Brzezinski et al. 1997).

The elimination half-life of cocaine has been reported to be dose-dependent, with non-linearity in cocaine plasma disappearance at plasma concentrations greater than 1.0 mg/L (Barnett et al. 1981). In single-dose studies the authors reported a half-life of 38 minutes following a 1 mg/kg intravenous dose and 87 minutes after a 3 mg/kg dose. The volume of distribution and the total clearance was increased at high dose. Dose dependent kinetics have also been demonstrated following intranasal administration, where elimination half-lives of 42 and 84 minutes were reported for a 64 and 96 mg dose respectively (Javaid et al. 1983). A capacity-limited pathway for cocaine metabolism, or inhibition of metabolism as metabolites accumulate, may account for the progressive decrease in cocaine clearance at higher doses (Barnett et al. 1981; Ambre et al. 1988).

In the presence of ethanol the hydrolysis of cocaine to BZE is inhibited (Harris et al. 2003). Harris et al (2003) compared the pharmacokinetics of cocaine when taken alone and when the same dose followed ethanol consumption. The half-life of BZE was similar under the two conditions (7 hours) although mean peak concentration in the plus ethanol condition (0.452 mg/L) was significantly lower than when cocaine was taken alone (0.652 mg/L). Mean urinary BZE in the plus ethanol condition was 69% of that when cocaine was taken alone. The co-consumption of cocaine and alcohol has also been associated with higher plasma concentrations of norcocaine (Farre et al. 1993). This might be attributed to the increased availability of cocaine for *N*-demethylation following ethanol ingestion.

The elimination of cocaine and its metabolites is prolonged substantially after chronic cocaine use. Weiss and Gawin (1988) periodically analysed the urine of long-term high-dose cocaine abusers ($n = 3$) following cessation of use and reported detection times ranging from ten to 22 days for BZE at a cut-off concentration of 0.3 mg/L. In naive users the detection time for BZE in urine was 1.5 – 3 days depending on route of administration (Saxon et al. 1988; Karch 2009). Moolchan et al. (2000) reported a half-life of 3.8 hours for cocaine in the plasma of chronic users also following abstinence. In some subjects the cocaine could still be detected in plasma 15 hours following last self-reported dose. In the urine of subjects who had reported using 1 – 12 g of cocaine per week for one to ten years, the presence of unmetabolised cocaine was confirmed through the first 4 - 5 days by GC-MS and for up to 15 days with radioimmunoassay (sensitivity = 0.5 ng/mL) (Cone and Weddington 1989). The prolonged presence of cocaine and metabolites in plasma and urine supports the theory that cocaine and metabolites accumulate in body tissues with subsequent slow release back into circulation (Cone and Weddington 1989; Burke et al. 1990).

It has been demonstrated that at any given dose level there is wide variability in half-life and detection period measured between subjects and within subjects across time (Jones 1984). This is largely attributed to the wide variability in phenotypes and genotypes of human esterases leading to wide inter-individual range in enzyme activity (Pantuck 1993; Wu et al. 2003). Diabetics have above average BuChE levels in their plasma (Abbott et al. 1993; Cucuianu et al. 2002) and might therefore metabolise cocaine even more rapidly than the average person.

2.3.5 Pharmacology

Cocaine has a stimulant effect on the CNS and increases heart rate and blood pressure as a graded function of dose following IV injection, insufflation, smoking or oral dosing (Foltin and Fischman 1991; Farre et al. 1993; Rush et al. 1999). The pharmacological effects are dose related and peak at times consistent with the appearance of peak blood concentrations (Cone 1995). However, cardiovascular effects and feelings of ‘high’ decline more rapidly than do cocaine blood concentrations and the exact same blood concentration that produced a ‘high’ when concentrations were rising can be associated with a dysphoric reaction when concentrations are falling (Javaid et al. 1978). The rush experienced by cocaine users follows a different time course than the cardiovascular

changes and drug tolerance begins to emerge after the first dose (Foltin and Haney 2004).

The stimulant effect of cocaine is largely mediated via inhibition of the presynaptic reuptake of the neurotransmitters, dopamine, norepinephrine, and serotonin at synaptic junctions (Karch 2007). The result is an increase in neurotransmitter concentrations in the synaptic cleft. Sympathetic stimulation by increased norepinephrine levels produces several physiological effects, including tachycardia, vasoconstriction, mydriasis and hyperthermia (Warner 1993). CNS stimulation results in increased alertness, energy and diminished appetite. The euphoria associated with cocaine use is believed to be related to the inhibition of serotonin and dopamine reuptake (Karch 2007). The strong reinforcing properties of cocaine have been linked with blockade of the dopamine transporter particularly (Ritz et al. 1987, Bergman et al. 1989), with chronic cocaine use reported to alter dopamine receptors (D1 and D2) and transporters (Seeman and Vantol 1994). Cocaine's anaesthetic properties are a direct result of its ability to block sodium channels in neuronal cells (Warner 1993). Like cocaine, COET also binds to the dopamine transporter blocking dopamine uptake and increasing extracellular dopamine concentrations (Hearn et al. 1991a; Jatlow et al. 1991). Unlike cocaine, COET appears to have relatively little action on norepinephrine or serotonin transporters (Hearn et al. 1991a; Bradberry et al. 1993). The combined use of cocaine and alcohol has been shown to induce greater subjective ratings of euphoria compared to when cocaine is taken alone (Farre et al. 1997; Hart et al. 2000). An increase in cocaine plasma concentration associated with this drug combination and the additive effect of COET are thought to be responsible for the enhancement of cocaine effects (Farre et al. 1997).

2.3.6 Toxicity

In comparison to the opiate class of drugs, deaths caused by cocaine alone are relatively uncommon (Baselt 2004). Death as a direct result of cocaine use is typically caused by seizures, cardiac arrhythmias or respiratory failure with cardiovascular complications accounting for the vast majority of cocaine related deaths (Karch 2005). Acute toxicity can manifest itself as cardiac standstill, but usually only at very high blood concentrations (> 20 mg/L) such as would be seen following rupture of cocaine packets in the bowels of body packers (Karch 2009). It has also been suggested that individuals with low cholinesterase activities become symptomatic of cocaine toxicity whereas

individuals with normal activity do not (Hoffman et al. 1992; Schwartz and Johnson 1996; Hoffman et al. 1998). Death in cocaine users is more commonly associated with long-term abuse of the drug. Chronic use initiates a number of neurochemical and anatomic changes in the heart and brain which favour sudden death even when post-mortem blood levels are low (Karch 2009). Cocaine is directly toxic to the myocardium (Peng et al. 1989). The hearts of chronic users undergo myocardial remodelling as a result of cocaine induced apoptosis (Kajstura et al. 2006) and increased production of calmodulin kinase II (Sun and Quamina 2004). Myocardial remodelling is characterised by an enlarged and fibrotic heart (Tazelaar et al. 1987; Brickner et al. 1991), features that are evident at autopsy (Karch and Stephens 1991).

Cocaine associated excited delirium (ED) is a syndrome associated with chronic cocaine use but with a very different clinical presentation to that of sudden death and massive overdose. It is characterised by an acute onset of delirium, agitation, combativeness, unexpected strength and elevated body temperature, often culminating in collapse and sudden death (Wetli et al. 1996; Mash et al. 2009). Whilst the stress response to restraint (i.e. in police custody) may be a contributory factor in manner and cause of death, a disturbance of dopamine release and transport within the CNS may ultimately be responsible for fatalities (Wetli et al. 1996; Mash et al. 2009). Neurochemical abnormalities involving the dopamine system have been identified in ED victims (Staley et al. 1994; Wetli et al. 1996).

There is also evidence that cocaine and norcocaine are hepatotoxic, whilst BZE and EME are not (Thompson et al. 1979). Norcocaine has been implicated in hepatic damage, with liver disease being a common finding in the autopsy of cocaine users (Karch and Stephens 1998). Although it had been thought that humans produce only minor amounts of norcocaine, and only following oral cocaine administration and/or when cocaine is co-administered with alcohol, Blaho et al. (2000) detected significant amounts in the plasma (0.030 ± 0.017 mg/L) of symptomatic cocaine abusers ($n = 111$) who had either smoked or injected the drug. This finding suggests that the reactive metabolite might play a greater role in human toxicity than once thought (Karch 2000).

At high doses cocaine depresses the respiratory centres and thus its use in combination with other CNS depressants, such as ethanol, opiates and benzodiazepines, could

increase the risk of overdose as a result of the additive depressant effect upon respiration (Dollery 1997). Evidence of a pharmacodynamic interaction of cocaine with heroin in cause of death has also been reported (Poletini et al. 2005). In a 2003 US survey of drug-related emergency room visits cocaine was detected in combination with alcohol (n = 25,049) five times more frequently than heroin and alcohol (n = 5160) (SAMHSA 2004). Ethanol is believed to potentiate the effects of cocaine on the cardiovascular system by inhibition of cocaine hydrolysis and formation of the active metabolite, COET (Farre et al. 1997; Hart et al. 2000; Laizure and Parker 2009). COET has more potent sodium blocking activity compared to cocaine and its proarrhythmic effects may be linked to sudden death as a result of ischemic myocardium (Xu et al. 1994). It has been reported that patients with detectable COET are more likely to require intensive care unit admission after trauma (Wiener et al. 2010).

2.4 Interpreting post-mortem drug and metabolite concentrations

There are several challenges in the interpretation of post-mortem drug and metabolite concentrations. The difficulty associated with correlating a specific blood concentration with toxicity is well known. In the first instance, drugs may affect different people in different ways and may even affect the same person differently on different occasions. The existence of tolerance introduces further complications in the interpretation of blood drug concentrations because a particular drug concentration may be associated with death in a naive user yet produce minimal symptoms in a tolerant individual. In addition, changes in drug concentration occurring during the post-mortem interval can grossly complicate the interpretation of toxicology findings.

In the determination of cause of death and likely level of drug impairment prior to death, toxicological measurements can never be considered in isolation. Autopsy findings and additional information such as that obtained from the scene, eye witness reports and the individuals' medical/drug history, where available, must also be taken into consideration.

2.4.1 Discerning heroin use

Because of its very short half-life heroin is rarely detected in biological samples. The primary metabolite, 6AM, is unique to heroin and thus its detection provides

unequivocal proof of heroin use (Meadway et al. 1998). Owing to the relatively short half-life of 6AM in blood it may only be detectable in this matrix for 2 - 3 hours following heroin exposure (Rook et al. 2006a). Tissues in which the window of detection for 6AM is extended, and in which the concentration of 6AM is typically higher than in the FB, include CSF (Pragst et al. 1999; Wyman and Bultman 2004), brain, spleen (Goldberger et al. 1994), VH (Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007), urine (Cone et al. 1991; Pragst et al. 1999) saliva (Jenkins et al. 1995) and hair (Goldberger et al. 1991; Kintz and Mangin 1995).

Codeine, present in heroin as an adulterant, both as codeine and acetyl codeine, is typically detected when an individual has consumed heroin (Gill and Graham 2002). It has been suggested that in the absence of 6AM the relative concentrations of morphine and codeine can be utilised to distinguish between morphine present as a result of heroin, and morphine metabolised from codeine following medicinal use of the latter (Lin et al. 1997; Gill and Graham 2002; Wyman and Bultman 2004). It has been demonstrated that finding a codeine to morphine ratio > 1 in blood provides evidence of medicinal codeine use (Nakamura et al. 1976). A ratio < 1 is suggested to be indicative of heroin use. In 20 6AM positive cases, Wyman and Bultman (2004) observed a codeine/morphine blood ratio of 0.059 ± 0.021 (range: 0.027 – 0.102). In VH ($n = 18$) the authors reported the mean ratio to be 0.199 ± 0.077 (range: 0.073 – 0.345). The utility of VH for distinguishing between heroin and codeine ingestion was also demonstrated by Lin et al. (1997) who examined 233 VH specimens and found that if morphine was derived from codeine and not heroin the ratio was always >1 (mean: 4.39 ± 2.95). The overall mean codeine/morphine ratio was 0.21 ± 0.11 .

Utilizing codeine to morphine ratios in the absence of 6AM for the determination of heroin exposure should be undertaken with caution since a low codeine/morphine ratio could also result from contamination of commercial morphine preparations (Baselt 2004) or as a result of poppy seed ingestion. The latter scenario may be distinguished by the analysis of thebaine, a naturally occurring opiate introduced into the body along with morphine and codeine following poppy seed ingestion (Meadway et al. 1998). However, the absence of thebaine cannot rule out poppy seeds as the source of morphine and codeine. Knowledge of witness reports, evidence collected at the scene

and indications of recent heroin use as determined at autopsy must all be considered when interpreting toxicological results.

2.4.2 Estimating survival time in heroin fatalities

Owing to the rapid metabolism of 6AM, its detection in blood can give an indication of survival time following heroin intake. Goldberger et al (1994) measured 6AM and morphine in 21 cases of heroin related death and calculated mean data separately for rapid (death occurred ≤ 15 minutes following administration), delayed (death several hours following administration) and undetermined deaths. Rapid deaths ($n = 8$) were characterised by a higher mean concentration of 6AM (0.019 mg/L; range: 0 – 0.083 mg/L; SD: 0.028) compared to delayed deaths ($n = 7$) (0.0067 mg/L; range: 0 – 0.030 mg/L; SD: 0.011). The authors also reported a greater likelihood of detection of 6AM in blood in rapid deaths (43%) compared with delayed (13%) and a lower mean concentration of 6AM in urine in rapid deaths (rapid: 0.20 mg/L; delayed: 0.78 mg/L). The lesser extent of urinary elimination in rapid deaths was also evidenced by the less frequent detection of heroin in this matrix.

The use of free morphine (FM) to total morphine (TM) concentration ratios as a means of evaluating the time of survival following heroin or morphine injection has been advocated in several studies (Garriott and Sturner 1973; Reed 1979; Spiehler and Brown 1987; Staub et al. 1990; Cone et al. 1991; Mitchell et al. 1991; Goldberger et al. 1994; Burt et al. 2001). An individual having a free morphine concentration that is relatively much higher than that of the conjugated morphine suggests insufficient time for metabolism and implies that death occurred quickly following drug intake. Staub et al. (1990) reported a mean FM/TM ratio in blood (site of collection not specified) of 0.76 (range: 0.5 – 1) in 44 individuals who died rapidly after heroin injection, whereas in 8 individuals who survived for an extended period the mean ratio was much lower (0.31). Goldberger et al. (1994) reported mean ratios of FM to total opiates in cardiac blood of 0.53 and 0.34 in rapid ($n = 8$) and delayed ($n = 7$) deaths respectively. In a larger study Spiehler and Brown (1987) found a FM/TM ratio in blood (site of collection not specified) of 0.68 in rapid deaths ($n = 56$) compared to an overall mean ratio of 0.42 ($n = 222$).

In an examination of 57 medical examiner cases Burt et al. (2001) reported lower femoral blood TM and FM concentrations in cases where 6AM was detectable in the blood. Comparing blood concentrations for cases with (n = 23) and without (n = 34) detectable 6AM demonstrated a mean TM concentration of 0.9 and 2.1 mg/L respectively with higher FM/TM ratios in individuals with detectable 6AM (0.35) compared to cases where the metabolite could not be detected (0.14). The presence of 6AM in blood combined with a high FM/TM ratio is thought to be indicative of recent heroin exposure (Burt et al. 2001).

Over-reliance on FM to TM ratios can, however, lead to erroneous conclusions with respect to survival time and some authors advise strongly against their use for this purpose (Skopp et al. 1996; Karch 2009). In addition to the time between drug administration and death, multiple factors influence the concentrations of the parent compound and its conjugated metabolites in post-mortem samples: 1) route of administration; 2) differences in V_d of morphine and its glucuronides; 3) variable hepatic and renal functions; 4) time between death and sampling (see section 2.5); 5) the tissue being sampled and; 6) individual/genetic differences, i.e. UDPG polymorphisms (Brunk and Delle 1974; Sawe and Odar-cederlof 1987; Osborne et al. 1990; Skopp et al. 1996; Moriya and Hashimoto 1997; Gyr et al. 2000; Karch 2009).

2.4.3 Relating post-mortem blood morphine concentrations to toxicity

One of the major problems in the interpretation of morphine concentrations is that the fatal concentrations reported in the literature often overlap the stated therapeutic (0.08 - 0.12 mg/L) and toxic concentrations (0.15 - 0.5 mg/L) (Uges 2004). Tolerance to both the pharmacological and respiratory depressant effects of morphine occurs rapidly and morphine concentrations obtained at autopsy may be misinterpreted if concentrations presumed to be fatal in non-tolerant individuals are applied to active heroin users or to individuals undergoing chronic pain treatment with opiates who have built up tolerance to the drug (Jung and Reidenberg 2005). In cases of acute overdose, blood morphine concentrations have ranged anywhere between 0.02 to 3.7 mg/L (Felby et al. 1974; Richards et al. 1976; Logan et al. 1987; Spiehler and Brown 1987; Sawyer and Forney 1988; Steentoft et al. 1988; Kintz et al. 1989; Goldberger et al. 1994; Logan and Smirnow 1996; Gerostamoulos and Drummer 2000; Burt et al. 2001; Jung and Reidenberg 2005; Crandall et al. 2006a). However, blood morphine concentrations in

excess of 1 mg/L have been reported in drug impaired drivers apprehended in Sweden (Jones et al. 2008). In patients receiving adequate opiate therapy for chronic pain, morphine concentrations as high as 2.1 mg/L have been observed (Tiseo et al. 1995). Heroin overdose as cause of death may be evident in cases involving heroin body packing, where blood morphine concentrations as high as 120 mg/L have been reported (Connett 1984; Joynt and Mikhael 1985).

In a great majority of heroin overdose cases the morphine concentrations recorded at autopsy are, in fact, lower than or similar to those recorded in living intoxicated addicts or heroin users who have died of causes other than overdose (Monforte 1977; Kintz and Mangin 1995; Zador et al. 1996). Darke et al. (1997) observed substantial overlap in the blood morphine concentrations measured in heroin overdose fatalities (mdn: 0.35 mg/L; 0.08 – 3.2 mg/L; n = 39) with those measured in living addicts receiving maintenance heroin (mdn: 0.09 mg/L; 0.05 – 1.45 mg/L; n = 100). Only four of the 39 heroin fatalities had morphine concentrations exceeding the highest concentration measured in the current users. Low blood morphine in cases of heroin overdose has been largely attributed to periods of abstinence resulting in loss of tolerance and/or the concomitant use of other drugs.

Research has shown that following a period of incarceration, and thus a period of drug abstinence or reduced use, there is an acute risk of drug-related death, particularly in the first few weeks following release (Verger et al. 2003; Binswanger et al. 2007; Farrell and Marsden 2008). These studies report the involvement of opiates in the vast majority of deaths. Significantly lower morphine concentrations have been reported in the hair of subjects who died from heroin overdose compared to hair from active users (Tagliaro et al. 1998; Darke et al. 2002). Since hair analysis gives an indication of retrospective drug use, the authors concluded that these findings were consistent with some abstinence from heroin use in the months preceding death.

An inverse relationship between blood alcohol and morphine concentration has been consistently reported in heroin/morphine fatalities suggesting that alcohol use decreases the amount of heroin required to induce a fatal overdose (Ruttenber and Luke 1984; Steentoft et al. 1988; Ruttenber et al. 1990; Fugelstad et al. 2003; Darke et al. 2007). In one study of heroin-related deaths it was determined that ethanol in excess of 0.1 g/dL

increased the probability of a heroin user experiencing a fatal intoxication by a factor of 22 (Ruttenber and Luke 1984). A trend toward lower morphine concentrations has also been reported in cocaine positive cases (Poletini et al. 2005). Table 4 summarises the results of seven studies that reported on the frequency of detection of morphine alone and morphine in combination with alcohol and/or benzodiazepines in cases of heroin overdose. The low percentage of morphine only cases reported in each study and the frequent detection of other CNS depressants, particularly alcohol (at mean concentrations in excess of 0.12 g/dL), highlights the association between heroin involved polydrug use and mortality.

Table 4 Percentage detection of alcohol and benzodiazepines and blood alcohol concentration in heroin related deaths

Study	N	Morphine only (%)	Benzodiazepine present (%)	Alcohol present (%)	Mean BAC (g/dL)	Range (g/dL)	SD
[1]	959	21	33	41	0.12*	0.01-0.78	0.13**
[2]	192	17	12	29	NR	NR	NR
[3]	152	27	27	45	0.14	0.01-0.35	0.08
[4]	23	13	9	74	0.16	0.03-0.28	0.072
[5]	100	23	12	68	0.14	0.02-0.42	NR
[6]	114	35	22	34	0.13	0.03-0.26	0.064

[1] Dark et al. (2010); [2] Fugelstad et al. (2002); [3] Zador et al. (1996); [4] Goldberger et al. (1994); [5] Monforte (1977); [6] Richards et al. (1976)

N: number of subjects; BAC: Blood alcohol concentration; SD: standard deviation

NR: Not reported; *median BAC reported; **result represents the interquartile range (no standard deviation reported)

As mentioned in Section 2.4.2, blood morphine concentrations depend on multiple factors. Whilst a very high morphine concentration may be consistent with the consequences of heroin toxicity, notwithstanding the issue of tolerance, the interpretation of low concentrations is more complicated. In addition to loss of tolerance and drug-drug interactions, a low blood morphine concentration may reflect an extended period between drug intake and death and this may be evidenced by the relative amount of metabolite. In the interpretation of blood concentrations an assessment of both free and total morphine is essential.

2.4.4 Relating post-mortem blood cocaine concentrations to toxicity

It is extremely difficult to correlate a specific blood cocaine concentration with toxicity and it is generally agreed that isolated measurements of post-mortem cocaine in blood cannot be used to assess or predict toxicity (Howell and Ezell 1990; Jenkins and Goldberger 1997; Karch et al. 1998; Blaho et al. 2000). Unlike alcohol intoxication, where specific blood concentrations can generally be related to physiological and psychological states, cocaine blood concentrations do not relate to symptoms. Blaho et al. (2000) described the toxicological findings in a large group of cocaine users who presented for treatment at an emergency department. They reported that there was no statistical correlation between cocaine or any metabolite concentration and the severity of clinical symptoms, incidence of adverse effects, outcome or need for interventional therapy.

In the past blood cocaine concentrations of more than 5 mg/L were thought to be uniformly fatal (Wetli and Mittleman 1981). However, the issue of tolerance has been highlighted in cases of trauma where cocaine concentrations well in excess of 5 mg/L have been recorded and yet clearly an unrelated finding (Karch et al. 1998). In a shooting case, where the victims' behaviour just prior to the incident was reported to be 'normal', the blood cocaine concentration measured at autopsy was 30 mg/L (Howell and Ezell 1990). In six cases where overdose as a result of ruptured gastrointestinal cocaine packets in body packers was evident, the maximum concentration measured in blood was 20.5 mg/L (Gill and Graham 2002). Thus, in the presence of very high cocaine levels, it may be difficult to distinguish between a case of massive cocaine overdose and a case of a highly tolerant individual where cocaine was merely an incidental finding.

Studies have shown the mean blood concentration of cocaine measured in cases of cocaine induced death to be two to six times higher than in cases where death was not attributed to the drug (Karch et al. 1998; Jenkins et al. 1999). Karch et al. (1998) reported a mean cocaine concentration of 0.487 mg/L in cases of death by lethal trauma with a range of 0 – 4.7 mg/L. In cocaine induced deaths the mean was 1.12 mg/L with a range of 0.001 – 18.1 mg/L. Jenkins et al. (1999) reported similar findings with means of 0.91 mg/L (0.05 – 3.4 mg/L) and 0.15 mg/L (0.03 – 0.5 mg/L) for cocaine induced and non-cocaine induced deaths respectively. In both studies the overall mean

difference was not found to be significant. Frequent overlap between the two groups, particularly over low to moderate concentration range, demonstrates the difficulty in assessing cause of death based solely on the blood cocaine concentration.

The majority of cocaine deaths encountered are not classic 'overdose' cases typified by high blood cocaine concentrations but instead are commonly due to the acute and chronic effects of cocaine (Jenkins et al. 1999). Cocaine induced deaths have been reported in chronic cocaine users following ingestion of only a few milligrams and therefore associated with very low blood concentrations (Smart and Anglin 1987; Jenkins and Goldberger 1997; Karch et al. 1998). Karch et al. (1998) observed striking physical differences between cocaine induced deaths and deaths where cocaine was an incidental finding. The cases of cocaine death presented with a significantly lower BMI and a significant increase in heart, lungs, liver and spleen when compared to the incidental finding group. These anatomic findings have been linked with chronic cocaine and stimulant abuse (Wetli et al. 1972; Brickner et al. 1991; Escobedo et al. 1992; Samanin and Garattini 1993; Karch et al. 1995). Cardiac alterations resulting from long-term cocaine use explain why equal blood cocaine concentrations may be lethal in some cases and innocuous in others.

Cocaine induced excited delirium is not necessarily associated with high blood cocaine concentrations. Mash et al. (2009) reported mean blood cocaine concentrations in excited delirium deaths (0.81 mg/L, n = 68) to be lower than that observed for non-psychotic cocaine deaths (3.3 mg/L, n = 100) and suggested this finding to be in keeping with longer survival times. Since the toxicology associated with agitated delirium deaths is similar to that observed in sudden death due to acute and chronic cocaine use, drug concentrations alone cannot differentiate between the two. Agitated delirium can be diagnosed by post-mortem neurochemical measurements, provided the brain is removed and processed within 12 - 18 hours (Wetli et al. 1996). Such neurochemical measurements are not routinely carried out in death investigations and knowledge of the victim's behaviour just prior to death and measurement of body temperature at the time of death is the best aid in making a diagnosis (Karch and Stephens 1998). Thus, in the interpretation of cocaine related fatalities, an assessment of the physical characteristics appears to be as, if not more, important than the assessment of blood toxicology.

2.4.5 Cocaine to metabolite ratios in the interpretation of cocaine fatalities

The use of cocaine to BZE metabolite ratios in blood appears to have little interpretative value (Spiehler and Reed 1985; Jenkins et al. 1999; Bertol et al. 2008; Karch 2009). Although Jenkins et al. (1999) reported a statistically significant elevation of the mean BZE blood concentrations in cocaine induced deaths (4.0 mg/L) relative to those recorded in non-cocaine deaths (0.89 mg/L) there was no remarkable difference between the average blood cocaine to BZE ratios measured in the two groups (0.21 in cocaine deaths versus 0.18 in non-cocaine deaths). The utility of blood cocaine to BZE ratios in the estimation of survival time is also not useful (Spiehler and Reed 1985; Bertol et al. 2008; Karch 2009). The V_d of BZE is much lower than that of cocaine so most of the cocaine will reside in the tissue and most of the BZE in the blood. An additional complication is encountered because the rapid chemical and enzymatic hydrolysis of cocaine in blood continues after death (Logan et al. 1997) further invalidating the use of cocaine/BZE ratios in determining survival time. Another consequence of this continued breakdown is that the concentrations of cocaine measured in blood at autopsy bear little resemblance to concentrations at the time of death (Logan et al. 1997) (discussed further in Section 2.5.2).

The interpretation of cocaine and BZE concentrations is thought to be most reliable in brain (Spiehler and Reed 1985; Stephens et al. 2004; Bertol et al. 2008; Karch 2009). Cocaine crosses the BBB rapidly (Misra et al. 1975; Nayak et al. 1976) and once in the brain is stable for much longer than in blood (Moriya and Hashimoto 1996) owing to the lack of esterase activity in this matrix (Satoh et al. 2002). Cocaine concentrations measured in brain are much higher than in blood and are thought to reflect more accurately the concentration at the time of death as compared with blood (Hernandez et al. 1994). Because BZE does not cross the BBB (Misra et al. 1975; Rowbotham et al. 1990) any BZE detected in brain is due solely to *in situ* formation (Spiehler and Reed 1985) and thus the cocaine to BZE ratio measured in brain is believed to give a good indication of survival time (Spiehler and Reed 1985; Stephens et al. 2004; Bertol et al. 2008; Karch 2009). A high brain concentration of cocaine but low or absent metabolite indicates recent exposure while the presence of BZE with little or no parent compound suggests a relatively remote use and/or chronic use (Stephens et al. 2004; Bertol et al. 2008).

Whereas cocaine and BZE ratios in blood cannot be used to assess lethality, results obtained in brain suggest that it is possible to make such a determination in this tissue. Bertol et al. (2008) found that in overdose cases ($n = 84$) the mean ratio of cocaine to BZE in the brain was $10 (\pm 5.8)$, over 14 times that measured in blood (0.69 ± 0.53). These results were clearly different from cases in which cocaine was an incidental finding ($n = 33$) where the mean ratios in brain and blood were $0.71 (\pm 0.61)$ and $0.21 (\pm 0.15)$ respectively. Similar results were obtained in an earlier study carried out by Speihler and Reed (1985). In 37 overdose deaths they reported mean cocaine/BZE ratios of 15 and 0.64 in brain and blood respectively. Where the presence of cocaine was incidental to death ($n = 46$) the ratios were 0.87 and 0.27 respectively. It appears that the assessment of brain cocaine and BZE concentrations can aid substantially in the interpretation of cocaine related death, both in cause of death determination and in the approximate estimation of survival time following drug intake.

2.5 Post-mortem change and redistribution

In addition to tolerance and poly-drug use, the interpretation of post-mortem blood concentrations is further complicated by the fact that drug concentrations measured at autopsy do not necessarily reflect the concentration at the time of death. It is known that after death there is a movement of drugs around the body which takes place via a process known as post-mortem redistribution (PMR) (Prouty and Anderson 1987; Pounder and Jones 1990; 1999). Drugs with a high V_d (> 1.0 L/kg) tend to be lipophilic and basic and concentrate into solid organs such as the lungs, liver and myocardium (Pelissier-Alicot et al. 2003). After death, as cell and tissue autolysis advances, cell membranes are broken down and drugs released from their binding sites can redistribute via passive diffusion from areas of high concentration in the tissues to areas of lower concentration in adjacent tissues and blood (Yonemitsu and Pounder 1993). In general the concentrations recorded in the central and heart vessels (pulmonary artery and vein and vena cava) are more subject to post-mortem increases than peripheral sites which are not in proximity to the central organs and gastrointestinal tract in which drugs accumulate ante-mortem (Anderson and Prouty 1989; Pounder and Jones 1990; Yonemitsu and Pounder 1993; Dalpe-Scott et al. 1995; Hilberg et al. 1999; Yarema and Becker 2005). Drugs sequestered in the pulmonary circulation can diffuse through the thin-walled pulmonary veins and significantly elevate concentrations in the left ventricle (Moriya and Hashimoto 1999).

As the post-mortem interval increases so does the likelihood that site- and time-dependent changes in drug concentrations will have occurred (Prouty and Anderson 1990). With time the peripheral blood achieves equilibrium with central blood (Fallani, 1961) and in cases of advanced decomposition the concentrations of drugs measured in the heart and peripheral blood are similar (Prouty and Anderson 1987). These authors also reported comparable central and peripheral concentrations following extensive resuscitation attempts. It is generally agreed that in order to avoid misinterpretation of cardiac blood drug concentrations which may have been falsely elevated post-mortem, blood samples should be collected from peripheral sites, which are less subject to change in the early post-mortem period (Anderson and Prouty 1989; Forrest 1993). The recommended site for sampling is the femoral vein and this should be cross-clamped proximally prior to sampling to avoid drawing blood with elevated drug concentrations from the proximal iliac vein and vena cava (Anderson and Prouty 1989; Forrest 1993; Pounder et al. 1996).

The advancement of putrefaction can contribute significantly to changes in drug concentrations. Invasion of the corpse by aerobic and anaerobic bacteria of the gastrointestinal tract, oral cavity and lungs is known to occur during putrefaction (Corry 1978; Melvin et al. 1984). These bacteria are capable of producing and/or metabolising many compounds in post-mortem blood and tissues (Corry 1978; Robertson and 1998; Moriya and Hashimoto 2005). It has been established that many drug metabolites are actively deconjugated by intestinal, bacterial enzymes resulting in enterohepatic recirculation and artefactual elevation of the free form of the drug (Sawyer and Forney 1988). Further, residual metabolic enzyme activity, variable with the nature of the enzyme involved, occurs in the early post-mortem period (Pelissier-Alicot et al. 2003). Continuing drug metabolism and metabolite deconjugation during the post-mortem interval is an important consideration when interpreting parent drug to metabolite ratios.

2.5.1 The post-mortem change and redistribution of morphine

The high V_d of morphine (3 – 5 L/kg) would indicate that it undergoes PMR and it has been shown to do so in animal models (Sawyer and Forney 1988; Koren and Klein 1992). There are, however, conflicting reports on its redistribution in humans. Gerostamoulos and Drummer (2000) found PMR not to be a factor in heroin fatalities. The authors examined 40 heroin-related deaths where the mean post-mortem interval

was 59 hours and found no significant difference between morphine concentrations in admission and autopsy blood. The cardiac to femoral blood concentration ratios averaged 1.1 for morphine, 1.3 for M6G and 1.1 for M3G. Logan and Smirnow (1996) found no evidence of time-dependent changes in morphine concentrations at either central or peripheral blood sites in 32 cases. They did, however, report consistently higher morphine concentrations in ventricular compared with femoral blood, with the greatest differences observed in cases where the ventricular morphine concentration exceeded 0.3 mg/L. In ten deaths involving morphine Dalpe-Scott et al. (1995) found central to peripheral blood morphine concentration ratios ranging from 1.0 – 5.8 with a mean of 2.2.

Other authors have also reported obvious differences between central and peripheral blood concentrations of morphine, M3G and M6G in humans (Prouty and Anderson 1990; Dalpe-Scott et al. 1995; Skopp et al. 1996; Bogusz 1997; Moriya and Hashimoto 1997). Concentration differences within the heart have also been reported for morphine with left ventricle concentrations two to three times higher than in the right ventricle (Moriya and Hashimoto 1997; Crandall et al. 2006a). It is thought that PMR from tissue to blood may easily double the morphine concentration in the latter (Skopp et al. 1996; Bogusz 1997) and since the glucuronides are predominantly distributed in plasma this occurrence would invalidate the use of morphine to metabolite ratios in estimating survival time (Skopp et al. 1996; Karch 2009).

Considering the low V_d of M3G (0.14 L/kg) and M6G (0.15 L/kg) (Hunt et al. 1999), these metabolites would not necessarily be expected to undergo PMR. However, Skopp et al. (1996) observed two to three fold differences in the molar concentrations of both glucuronides in blood sampled from different sites in four heroin overdose deaths. Some of the variation was attributed to variations in water content (65 - 83%) and haematocrit (25 – 75%). According to Carrupt et al. (1991), morphine glucuronides can exist in two conformational forms, the folded one being more lipophilic than the unfolded one. The site- to site-variations in the concentrations of these metabolites could be associated with this particularly.

The concentrations of free morphine can increase significantly in the post-mortem period due to hydrolysis of the morphine glucuronides (Moriya and Hashimoto 1997).

Escherichia coli, one of the most predominant bacteria present in intestinal flora, is an important source of β -glucuronidase (Fish and Hayes 1974; Zezulak et al. 1993), a hydrolase known to deconjugate morphine glucuronides, particularly M3G (Romberg and Lee 1995), in putrefying blood and tissues (Moriya and Hashimoto 1997; Carroll et al. 2000; Skopp et al. 2001b). The hydrolysis of morphine glucuronides back to free morphine during the post-mortem interval can alter the ratios significantly as a function of time.

2.5.2 The post-mortem change and redistribution of cocaine

Cocaine concentrations can change significantly during the period between death and autopsy (Hearn et al. 1991b). Several authors have reported significant variation in blood cocaine concentrations according to site and time of collection. One reason for such variation is the persistence of the cocaine-metabolising system in the post-mortem interval (Logan et al. 1997). Continuing hydrolysis due to residual esterase activity in the post-mortem interval is supported by the in vitro observation of increasing EME concentrations in blood (Baselt 1983; Isenschmid et al. 1989). Chemical hydrolysis of cocaine to BZE and of EME to ECG also continues at or near physiological pH (Isenschmid et al. 1989).

After death, continued anaerobic metabolism and the onset of putrefaction can reduce the pH of the blood to pH 5.5 – 6.0 (Logan and Peterson 1994). This acidosis dramatically slows the rate of chemical hydrolysis of cocaine to BZE, and of EME to EGC (Stewart et al. 1977; Baselt 1983; Isenschmid et al. 1989). Because esterase mediated hydrolysis of cocaine to EME continues, the amount of EME in post-mortem blood increases while the cocaine concentration decreases (Logan and Peterson 1994). The metabolic profile observed in post-mortem blood may therefore bear little resemblance to that at the time death and explains why estimations of survival time based on cocaine and metabolite concentrations in blood is not possible. In highly putrefied specimens ECG may be the only indicator of cocaine use following extensive post-mortem hydrolysis (Skopp et al. 2001a).

Further complicating the pattern of post-mortem change, the pharmacological properties of cocaine (weakly basic with a V_d of 1.9 - 2.4 L/kg; Hart et al. 2000) would indicate that it is also subject PMR. The site- and time-dependence of cocaine in post-mortem

blood has been reported (Hearn et al. 1991b; McKinney et al. 1995; Logan et al. 1997) Hearn et al. (1991b) examined seven autopsy cases and found that cocaine concentrations usually increased over time in cardiac and femoral vein blood (positive concentration difference), but decreased in subclavian vein blood. This data is presented in Figure 3. From the figure it can be seen that in femoral and cardiac blood there is no relationship between the size or direction of concentration change and the length of post-mortem interval. In an examination of 19 autopsy cases, Logan et al. (1997) also found no consistent pattern of direction or magnitude of change in femoral and cardiac blood concentrations of cocaine, BZE and COET. The competing process of tissue release and continuing chemical and enzymatic breakdown of cocaine and its metabolites post-mortem are believed to contribute to the observed differences in the concentration of cocaine and its metabolites both between sites and over different sampling intervals (Hearn et al. 1991b; McKinney et al. 1995).

2.6 Alternative tissues

Depending on the post-mortem interval specimens that would normally be collected at autopsy i.e. blood and urine, may not be available for collection. In such instances the toxicologist must rely on analysis of remaining tissues less affected by putrefaction. For drugs that are broken down by hydrolytic esterase activity, e.g. cocaine and 6AM, tissues that lack esterase activity might, in fact, provide more reliable specimens for toxicological interpretation compared with blood. This has been well demonstrated for brain in the measurement and interpretation of cocaine. VH and skeletal muscle may also provide more reliable toxicological specimens because, like brain, they lack esterase activity and are located away from central visceral tissues, in which basic, lipophilic drugs accumulate. Consequently, these tissues should be less affected by post-mortem change and redistribution compared with blood.

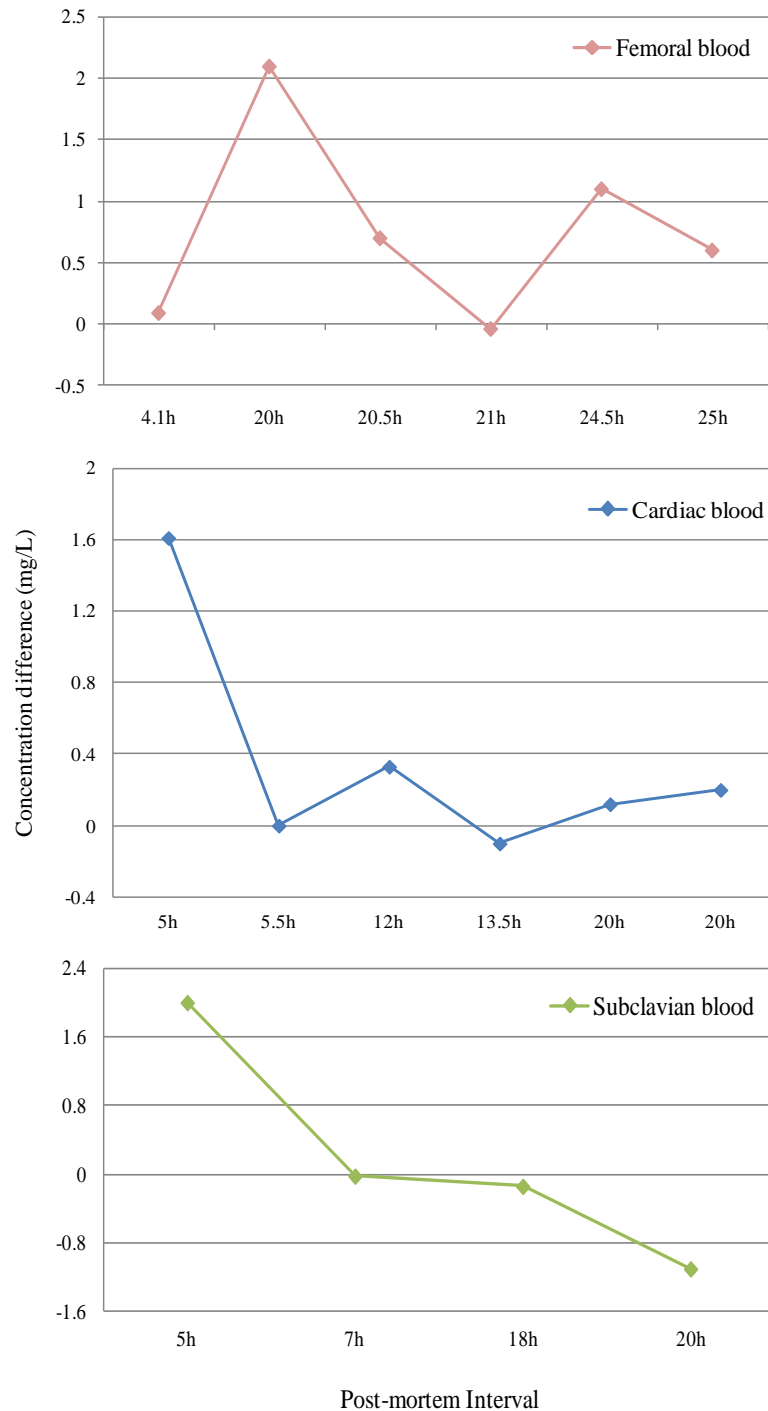


Figure 3 Concentration difference (mg/L) between scene and autopsy cocaine concentration in femoral blood, cardiac blood and subclavian blood in cases with varying post-mortem intervals.

Positive concentration differences represent an increase in blood concentration with time (autopsy concentration > scene concentration).

(adapted from Hearn et al. 1991b)

2.6.1 Vitreous Humour

It has been demonstrated that VH is useful to screen for a variety of drugs (Maurer 1999). VH has several advantages as a toxicological specimen: 1) it is composed of 99% water thus providing a relatively clean specimen for analysis. The remaining 1% is made up of sugars, salts, phagocytes and a network of collagen fibres (Forrester et al. 1996); 2) the eye is located well away from the central body cavity and hence drugs in the vitreous are less likely to be influenced by post-mortem changes; 3) esterases are absent in the VH and thus this matrix offers an extended window of detection for drugs broken down via hydrolytic esterase activity (Antonides et al. 2007).

The VH constitutes 80% of the eye and has a volume of approximately 4 mL (Tripathi and Tripathi 1984). The pH of the VH is 7.5 (Levine and Jufer 2008). The human eye is supplied with an extensive network of blood capillaries hence drugs circulating in the blood may pass into the VH. Equilibrium between blood and vitreous is slower than between the blood and other extracellular fluids suggesting the presence of a barrier, named the blood-vitreous barrier (Levine and Jufer 2008). The movement of molecules in and out of the VH occurs by a number of mechanisms. For low-molecular weight molecules, e.g. common drugs, diffusion has been cited as the major method of drug transport from systemic circulation to the VH (Gardener 1987). Only free drug is able to leave the blood and enter the VH so highly protein bound drugs would not be expected to have significant concentrations in the VH. Small lipophilic compounds should penetrate readily into the VH and the drug concentration in the VH should follow the blood concentration with a certain delay at a concentration ratio $C_{\text{VH}} / C_{\text{blood}}$ as determined by the protein binding (Pragst et al. 1999). After direct injection into the VH the elimination of lipophilic drugs occurs quickly by diffusion through the membranes, whereas hydrophilic drugs are more slowly eliminated together with the fluid streams (Larsen et al. 1991). Water movement out of the VH is significant as approximately 50 % of the water is replaced every 10 – 15 minutes (Levine and Jufer 2008).

VH is particularly useful for ethanol analysis. Studies have shown the mean VH/blood ratio of ethanol is very close to values expected from the distribution of water in these two biological specimens, namely 1.15 – 1.24: 1 (reviewed by Kugelberg and Jones 2007). The data available on the relationship between drug concentrations in blood and VH does not compare to the scores of publications that have compared and contrasted

ethanol concentrations in these matrices. Thus the main disadvantage of VH in drug analysis is the limited interpretive value of analytical results. The relatively small amount of studies that have compared blood and VH opiate and cocaine concentrations in a moderate to large sample number are discussed in the following sections.

2.6.1.1 Opiates

Vitreous is a particularly important matrix for discerning heroin use. Although urine is generally considered the best specimen for 6AM detection (Rook et al. 2006b) it is not always available at autopsy. Owing to the lack of esterase activity in VH, 6AM has been shown to persist in this matrix longer than in blood and concentrations of the drug in VH consistently exceed those blood (Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007). In 36% of cases (n = 25) investigated by Wyman and Bultman (2004) and in 50% of cases (n = 12) investigated by Antonides et al. (2007) 6AM was detected in VH but not in blood. In each of these cases, heroin exposures would not have been detected without the assay of VH. The utility of VH for differentiating between codeine and heroin induced fatalities in the absence of 6AM has already been discussed in Section 2.4.1.

In contrast to 6AM, the concentrations of morphine in VH are typically reported to be lower than the corresponding FB concentrations (Pragst et al. 1994; Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007). In an investigation of 154 opiate-related fatalities, Pragst et al. (1994) reported that VH to FB ratios were frequently in the range 0.4 – 0.6 for free morphine. These authors suggested that the survival time significantly influenced VH/FB morphine concentration ratios with lower ratios thought to be a reflection of incomplete distribution in cases with a short survival time. In cases in which the survival time was known to be greater than 5 hours, concentrations of morphine observed in VH were higher than in FB. The authors also suggested the typically lower concentrations of morphine in VH versus FB to be the result of the relatively high plasma-protein binding of morphine (20 - 35%) (Moffat et al. 2004) leading to smaller equilibrium concentrations in VH. Pragst et al. (1994) also found the concentration of conjugated morphine to be much lower in the VH than in the blood with VH/FB ratios < 0.3. Codeine concentrations are reported to be comparable to that measured in FB (Wyman and Bultman 2004).

Wyman and Bultman (2004) examined 25 cases and found a mean VH/FB concentration ratio of 0.36 ± 0.18 (standard deviation) for morphine. The authors suggested the low standard deviation to reflect a correlation between the two matrices. In the same study a mean VH/FB ratio of 11.3 ± 9.4 ($n = 13$) and 1.03 ± 0.39 ($n = 25$) were reported for 6AM and codeine respectively with a poor correlation for 6AM evidenced by the high standard deviation. Scott and Oliver (1999) examined 20 morphine positive cases and observed a correlation coefficient (r) of .697 between the concentration of morphine in VH and blood. The authors reported the correlation to be dependent on the rapidity of death since a higher correlation ($r = .885$) was obtained when only sudden deaths were taken into account ($n = 17$). No correlation was observed for 6AM ($r = .006$, $n = 20$; sudden death $r = .03$, $n = 17$). One of the problems with this study, in addition to the small sample number, is that the site of blood collection was not stated. Further, the analytical method did not appear to have been validated and so the accuracy of the reported data cannot be confirmed. In fact, the 6AM concentration data reported by these authors are not what would be expected given the ranges generally reported in the scientific literature. In blood the concentration of 6AM is typically less than 0.1 mg/L (Chapter 7; Goldberger et al. 1994; Wyman and Bultman 1999, Fugelstad et al. 2002) yet Scott and Oliver reported blood concentrations as high as 0.51 mg/L with a mean close to the maximum concentration typically reported by other authors (0.096 mg/L, $n = 20$). The concentration of 6AM measured in the VH is typically reported to be higher than in the blood but in general is still < 0.2 mg/L (Chapter 7; Pragst et al. 1999; Wyman and Bultman 1999; Fugelstad et al. 2002). Scott and Oliver reported a mean 6AM concentration of 0.24 mg/L in the VH with five cases having a 6AM concentration > 0.4 mg/L. The maximum concentration in this matrix was reported to be as high as 1.7 mg/L. Since the method was apparently not validated, the accuracy of these results is questionable.

In a larger study Stephen et al. (2006) measured morphine in VH and FB in 52 cases and observed a Pearson's correlation coefficient (r) of .716 ($p < .001$). The authors fitted a linear equation to the observed data to enable the prediction of FB morphine from the VH concentration and concluded that where blood is unavailable at autopsy the concentration of morphine in VH can be used to predict the concentration of morphine in FB. The implication that the spread of the data would have on the ability of the equation to accurately predict morphine concentrations in blood was not discussed. In a

series of 40 heroin related deaths Gerostamoulos and Drummer (1997) reported a Pearson's correlation coefficient (r) of .261 for morphine in FB and VH and suggested that VH morphine concentrations may be of limited use for toxicological interpretation.

Opiate concentration data in VH is still relatively limited and the assessment of the relationship with FB concentration is too often based on small sample numbers or individual cases. Further investigations using large sample numbers are required to clarify the relationship between opiate concentrations in these two matrices so that the interpretive value of VH might be better understood.

2.6.1.2 Cocaine

Although conversion of cocaine via hydrolytic enzyme activity does not occur in the VH it is likely that spontaneous chemical hydrolysis to BZE does. This spontaneous conversion has been demonstrated to occur above pH 6.0 (Isenschmid et al. 1989). The pH of VH is 7.5 and thus formation of BZE would be expected in this matrix. Nevertheless, as with 6AM the window of detection for cocaine is extended in this matrix (Mackey-Bojack et al. 2000; Duer et al. 2006; Antonides et al. 2007). In 13% of cases ($n = 40$) analysed by Antonides et al. (2007) and in 27% of cases ($n = 62$) analysed by Mackey-Bojack (2000) cocaine could be detected in the VH but not in the blood. Antonides et al. (2007) also reported that in 28% of cases where acute intoxication was apparent just prior to death, and where the post-mortem interval was short, the concentration of cocaine in the blood exceeded that in the VH. In these cases BZE was significantly higher in blood by as much as 10-fold. The authors proposed this observation to be indicative of a lag in the distribution of cocaine and BZE into the vitreous.

In the 62 cases examined by Mackey-Bojack et al. (2000) the mean concentration of cocaine in VH (0.61 mg/L) was slightly higher than in FB (0.49 mg/L) although the difference was not significant. There was also no significant difference in the mean concentration of COET measured in the VH (0.027 mg/L) and FB (0.022 mg/L). The mean concentration of BZE in VH (0.99 mg/L) on the other hand was found to be significantly lower than in FB (1.9 mg/L). The authors reported a linear relationship between the concentrations of cocaine ($r = 0.85$) and BZE ($r = .763$) in VH and FB. The correlation for COET was somewhat lower ($r = .433$). The authors also noted that mean

concentrations of BZE in FB and VH were higher in cases where ethanol was present compared to when ethanol was absent. Because the concentrations of COET in VH and FB were not well correlated the authors concluded that VH may not be a reliable specimen for the quantitative measurement cocaine and its metabolites. Logan and Stafford (1990) reported a coefficient of determination (R^2) of .70 for cocaine in FB and VH in 28 cocaine positive cases but with considerable spread in the data. The authors concluded that the wide range and poor correlation limits the use of VH cocaine concentrations in assessing corresponding blood concentrations. The use of VH cocaine concentration in the prediction of blood concentrations is not thought to be reliable because the magnitude and direction of the concentration differences between the two specimens is too variable (Mackey-Bojack et al. 2000).

There is evidence that a more reliable relationship may exist between the concentration of cocaine in blood and VH when the total sums of the concentrations of cocaine and its hydrolysates (BZE, EME and ECG) are measured (Duer et al. 2006) In measuring the concentrations of molar sums, Duer et al. (2006) observed high correlations between VH and FB ($r = .939$). The authors excluded alcohol positive cases from their study and did not discuss the potential effect of COET formation upon the molar sum of the cocaine products. In contrast to Antonides et al. (2007), who suggested that the distribution of cocaine and BZE from the blood into the VH occurs with some delay, Duer et al. (2006) proposed that the high correlation observed between blood and VH was indicative of rapid distribution of cocaine and its hydrolysates into the VH.

There is evidence that the vitreous humour may not be so well isolated from the effects of PMR, particularly with respect to cocaine concentration. In a case report, Beno and Kriewall (1989) observed a 330 % increase in vitreous humour cocaine concentration over a 19 hour interval. Significant increases (300%) in VH cocaine concentrations have also been noted in dosed juvenile swine 8 hours following death (McKinney et al. 1995). In an investigation of PMR in a human decedent, Hearn et al. (1991b) measured the VH concentrations in one eye just after death and in the other 18 hours later. The cocaine concentration had risen from 1.0 mg/L at the time of death to 3.5 mg/L after 18 hours. The concentration of BZE also rose, but to a lesser degree, from 1.1 to 1.7 mg/L. The corresponding femoral blood concentration for cocaine and BZE respectively were 1.8 and 3.6 at the time of death and 3.9 and 8.1 mg/L when sampled from the same vein

after 20 hours. The post-mortem increase of cocaine observed in VH is thought possibly to be due to release from the ocular tissue or brain (Skopp 2004). In a dog model the choroid-retina showed the highest level of digoxin and the drug leached into VH with time (Binnion and Frazer 1980). An increase in MDMA concentrations in the VH in a rabbit model has been attributed to release of drug sequestered in the globe wall (De Letter et al. 2000).

Whilst the increased stability of cocaine in the VH promotes its use as a toxicological specimen for the determination of this drug, the observation of increasing cocaine concentration during the post-mortem interval might limit its interpretive value. The collection of additional cocaine and metabolite data in VH is required to clarify its interpretive value.

2.6.2 Skeletal muscle

Skeletal muscle as a post-mortem specimen has potential advantages. Whereas blood and other body fluids may be missing in burned, putrefied, mummified or exhumed bodies, muscles tissue is usually available and often well preserved (Christensen et al. 1985). Muscle is remote from the central body compartment and therefore less likely to show elevated drug concentrations attributable to circulatory post-mortem changes. Further, esterases responsible for the breakdown of ester-containing drugs are not present in muscle tissue (Sato et al. 2002). A further advantage of muscle tissue as a specimen for drug determination relates to its low post-mortem pH.

Upon death there is a rapid decline in muscle pH as a result of post-mortem metabolism and conversion of glycogen to lactic acid. In cattle the pH has been reported to drop from pH 7.1 at the time of death to pH 5.4 - 5.7 18 - 24 hours after death and then to slowly rise again to around pH 6.5 at which point the tissue is beginning to decompose (Lawrie and Ledward 2006). The situation in human skeletal muscle appears to be comparable with post-mortem pH values ranging from 5.7 – 6.0, depending on the muscle (Langford et al. 1998). Slightly higher pH values were recorded in cases with an extended PMI, particularly if the body had not been refrigerated. Post-mortem pH values for thigh muscle were typically around pH 6.0 (Langford et al. 1998). Drugs that undergo spontaneous chemical hydrolysis at alkaline pH should therefore have increased stability in muscle tissue during the post-mortem interval.

Moriya and Hashimoto (1996) found cocaine and COET to be substantially more stable in muscle compared with blood. Following storage at 20 – 25 °C, $26.2 \pm 19.4\%$ of the original cocaine concentration could still be detected in muscle after 5 days, whereas in blood cocaine was undetectable within 1 day. The increased stability of cocaine in muscle has been further indicated by its detection in decomposed human muscle at concentrations ranging from 0.003 to 1.5 mg/kg (Manhoff et al. 1991). The stability of morphine in post-mortem muscle has been evidenced by the case of Dr Harold Shipman, in which ten bodies were exhumed, some of which had been embalmed, as much as 4 years after burial (Pounder 2003). Morphine was detected in the muscle in each of these cases with TM and FM concentrations in the range 0.2 – 1.0 mg/kg and 0.3 – 0.7 mg/kg respectively. Despite the advantages of this matrix for drug detection, the amount of data available for opiates and cocaine in muscle data is extremely limited and as such a relationship between blood and muscle has not been clearly established for opiates or cocaine.

2.6.2.1 Drug distribution in skeletal muscle

Garriott et al. (1991) compared drug concentrations in thigh muscle (sampling site not defined) and aortic blood in 32 cases where, in most instances, death was not drug related. The authors reported that for most common basic drugs the muscle to blood ratios were often near unity and, therefore, suggested muscle to be a useful quantitative alternative to post-mortem blood. For all drugs analysed the authors noted that the muscle to blood ratios seemed to be dependent on the time course between ingestion and death, with low ratios associated with rapid death, and hypothesised that the muscle to blood ratio may be used as an indicator for such estimations. The main problem with this study is that cardiac blood was used and this is known to be subject to post-mortem redistribution. The authors did not discuss the potential contribution of post-mortem elevation of drug concentrations in cardiac blood to the low ratios observed in rapid death cases. Further, the authors did not mention the validation of the analytical methods employed and thus the validity of the reported data cannot be confirmed.

In eight cases in which Garriott et al. (1991) detected cocaine and its major metabolites, BZE and EME, the mean muscle to aortic blood ratio for cocaine was 1.2 with a range of 0 - 6.5. In two cases of acute cocaine overdose the authors reported very low

muscle/blood ratios of 0.1, believed to be consistent with rapid death and insufficient time for tissue equilibration. Consistently low ratios were reported for BZE (mean: 0.26; range: 0 - 0.7) and EME (mean: 0.11; range: 0 - 0.34). In three cases in which BZE was not detected in the muscle, the corresponding cocaine concentrations were 0.06, 0.15 and 1.1 mg/kg, and thought to be indicative of insufficient time for tissue equilibration of BZE. In cases in which morphine (n = 9) and codeine (n = 11) were detected in muscle, the morphine concentrations were found to be approximately equivalent to that in the blood (mean muscle/blood ratio: 1.4; range: 0 - 3.6). Codeine, on the other hand, was found in very low relative concentrations in muscle (mean muscle/blood ratio: 0.16; range: 0 - 0.7) (Garriott 1991). It has been suggested that muscle may provide a good specimen for roughly predicting the ratio of free to total morphine in blood (Moriya and Hashimoto 1997). In a single case of rapid death following injection of heroin and methamphetamine, these authors reported free to total morphine ratios of 0.86, 0.74 and 0.79 in the femoral blood, femoral muscle and cardiac muscle respectively.

In a study by Christensen et al. (1985) drug concentrations in skeletal muscle (sampled from the quadriceps or biceps), extremity vein blood (not otherwise specified) and liver were compared in 142 cases of fatal drug overdoses. The authors reported the concentrations of acidic drugs in muscle to be of the same magnitude as that in blood and of a similar magnitude for basic drugs (mainly tricyclic antidepressants). However, in a single case where cocaine was present, the concentration in the muscle was eight times higher than in the blood. In three overdose cases, one of which was a morphine overdose, the authors assessed site-dependency in drug concentration by sampling each of the four extremity muscles and the corresponding venous blood. The authors found the drug concentrations in muscle to be independent of the site of sampling and to be as consistent as those found in the corresponding blood samples. Based on their findings the authors suggested that the minimum ingested dose could be estimated by multiplying the drug concentration in an extremity muscle by the body weight and the fraction of the body weight made up by the muscle mass. However, a within case variability of drug concentrations has since been confirmed (Williams and Pounder 1997; Langford et al. 1998) and has adverse implications for the proposed estimation of ingested drug dose.

Williams and Pounder (1997) examined the homogeneity of drug concentrations within thigh muscle in eight fatal overdose cases. Ten-30 random samples were taken and analysed for selected tricyclic antidepressants, benzodiazepines, paracetamol and dothiepin. They reported %RSDs ranging from 10.5 for carbamazepine (range: 10.3 - 17.0 mg/kg) to 50 for thioridazine (0.3 - 2.9 mg/kg). The large variation observed for each of the drugs indicated that skeletal muscle was not homogenous with respect to drug concentrations in fatal overdose cases. The RSD was found to be lower for drugs with a lower V_d . The authors also reported significant variation in muscle to femoral blood drug ratios thought potentially to be influenced by survival time. It should be noted that the authors did not report on the characterisation of the analytical methods employed to analyse muscle or blood and if assays had not been fully validated then the reliability of this data may be questionable.

Langford et al. (1998) examined drug concentrations in twelve named muscles in eleven overdose cases and three cases of chronic therapeutic drug use. The drugs measured were predominantly benzodiazepines, amitriptyline and paracetamol. The authors reported marked within-case variability in drug concentration and muscle to femoral blood ratios varied substantially for each muscle. With the exception of the diaphragm, in which drug concentrations were invariably higher than all other muscles, there was no clear pattern, although drugs with a higher V_d showed the greatest variation. In two cases involving the chronic therapeutic use of temazepam the authors reported comparable concentrations in several peripheral muscles possibly indicative of tissue equilibration in chronic users. However, in a single case involving the chronic use of fluoxetine, the concentration range was large (0.03 – 0.6 mg/kg; %RSD = 56%). The difference in the distribution of these two drugs was attributed to the V_d of temazepam (0.8 - 1.0 L/kg) being much lower than that of fluoxetine (V_d = 20 - 42 L/kg). The authors found that in general the lower the V_d of an analyte the lower the variation in drug concentration. As with the studies conducted by Garriott et al. (1991) and Williams and Pounder (1997), no method validation data was reported for any matrix. It was simply stated that the assays employed followed the standard investigative procedures used and developed in house.

Based on these most recent studies, it has been concluded that muscle is of use for qualitative drug determination but not for the quantitative corroboration of a blood

sample or as a quantitative alternative to blood (Williams and Pounder 1997; Langford et al. 1998). However, the theory that a muscle to blood drug concentration ratio might provide insight into time between drug ingestion and death, as suggested by Garriott et al. (1991), should not be discounted but rather explored more systematically using appropriately validated methodology.

2.7 Problems with the current tissue distribution data

A major problem with the drug distribution data that is currently available relates to the lack of consistency of blood and tissue sampling sites. Direct comparison of the data is complicated since sampling sites and blood collection techniques are varied. In some cases the details of specimen collection, such as whether cardiac or femoral blood was sampled, are omitted. Even when the use of femoral blood is stated, it is not always known whether the vein was cross-clamped proximally. If the vein is not ligated there is a risk of drawing central blood with elevated drug concentrations into the sample (Anderson and Prouty 1989).

In the relatively small number of case reports where drug concentration has been measured in muscle tissue, the sampling site is not clearly defined. The sampling site may, for example, be described as 'thigh muscle' or 'extremity muscle', yet it has been demonstrated that there is great inter-muscular variation in drug concentrations (Williams and Pounder 1997), including between different thigh muscles (Langford et al. 1998). Unless the sampling site is clearly defined, evaluating trends in the drug concentration data from muscle is impossible.

A further issue which has been raised previously in this chapter relates to the validity of the analytical methods employed to obtain tissue distribution data. Method validation is an essential component of the measure that laboratories should employ to ensure that they produce accurate and reliable results. A common finding in the tissue distribution literature is the failure to mention whether the assays were validated prior to use. In such instances the assumption is that the method was not validated, and this raises questions as to the reliability of the reported data. In order to assess the interpretive value of a given tissue, the employed analysis must, at a minimum, provide accurate and reproducible results.

2.8 Drug stability during sample storage

Degradation mechanisms occurring during autolysis and putrefaction may also be seen during sample storage. Thus, the potential for drug concentrations to change following specimen collection is a further factor to be considered when interpreting results. The method of sample storage and preservation is vital particularly when analyses cannot be performed promptly. Data on the stability of opiates and cocaine in fluids and tissues other than blood, plasma, serum and urine are rare. Even in blood, very little data has been reported on the stability of 6AM.

2.8.1 Opiates (heroin and metabolites)

The rate of deacetylation of diamorphine and 6AM has been shown to be pH and temperature dependent in aqueous solution with the rate of degradation substantially increased at higher pH and temperature (Nakamura et al. 1975; Beaumont 1982; Barrett et al. 1992; Romolo et al. 2003). Diamorphine is rapidly and spontaneously hydrolysed at alkaline pH forming 6AM as the major product and morphine as a minor product (Barrett et al. 1992). The rate of this degradation was reduced considerably at pH 5.6 and pH 4.0 with the half-life of diamorphine reported to be greater than 14 days at temperatures of 4, 25 and 37°C. The rate of deacetylation of 6AM to morphine was consistently slower than that of diamorphine to 6AM under identical pH and temperature conditions. In an investigation of 6AM stability in hair extraction incubation media, 6AM declined by 6.3% and 33% in phosphate buffer solutions of pH 7.0 and 8.0 respectively following incubation at 45°C for 18 hours (Romolo et al. 2003). The authors also reported substantial hydrolysis (46.4% and 33.8%) in aqueous acidic media (0.1 N and 0.01 N HCl respectively) following incubation. Under the same incubation conditions, Poletini et al. (1997) observed comparable 6AM hydrolysis (47%) in 0.1 M HCl.

In biological fluids, diamorphine is subject to rapid hydrolysis by esterases and is completely deacetylated within 20 minutes in human blood at room temperature (Nakamura et al. 1975; Lockridge et al. 1980; Barrett et al. 1992; Romolo et al. 2003). Barrett et al. (1992) demonstrated diamorphine to be rapidly degraded to 6AM in plasma with a half-life of 354, 18 and 3 minutes at a temperature of 4, 25 and 37°C respectively. Owing to the instability of diamorphine in biological samples, accurate determination of the drug is only really achieved with rapid stabilization, i.e. freezing

the sample in liquid nitrogen with subsequent extraction using reagents cooled to 4°C (Barrett et al. 1992). Low pH, addition of NaF and freezing at -20°C is only partially effective in stabilizing heroin in blood (Karinen et al. 2009).

In whole blood, plasma BuChE, as well as erythrocyte AChE, have been shown to hydrolyse heroin to 6AM with further hydrolysis to morphine mediated by AChE but not BuChE (Lockridge et al. 1980; Salmon et al. 1999). Owing to the absence of AChE in plasma, deacetylation of 6AM to morphine is not observed in this matrix (Nakamura et al. 1975; Smith and Cole 1976; Barrett et al. 1991; Barrett et al. 1992). The stability of 6AM in blood under different storage conditions is not well documented. It is known that 6AM degrades in a blood sample at room temperature with a half-life of 8 hours (Pichini et al. 1999). At 4°C 6AM was reported to be unstable in blood with only 20% of the initial concentration detected after storage for 7 days (Boy et al. 2008). The authors demonstrated that in dried blood spots the stability of 6AM was approximately doubled. The only information available regarding the effect of preservative on 6AM stability in blood is that provided by Wathanafa and Cooper (2010), who reported that the addition of NaF slowed but did not prevent the breakdown of 6AM in blood stored at room temperature.

Two studies have provided limited data on the stability of 6AM in VH. Pragst et al. (1999) added 6AM to human VH and found no significant degradation over a 7 day period when stored at 0 and 4°C. Holmgren et al. (2004) stored four 6AM positive VH samples from real cases with and without potassium fluoride (KF) at -18°C. On re-analysis 1 year later the authors reported that 6AM could only be detected in the samples to which KF had been added. No concentration data was reported for 6AM so the amount of degradation that occurred over the 12 month period is unknown. The significance of this report is further limited by the small sample number investigated and the assessment of only one temperature and time point. Thus, the stability of 6AM in VH stored with and without the addition of preservative requires further elucidation.

The stability of morphine in pharmacological preparations shows varying degrees of degradation, particularly in aqueous solution, with the formation of pseudomorphine as a major product and morphine-*N*-oxide and apomorphine as minor products (Vermeire and Remon 1999). These authors reported the rate of chemical oxidation to be

accelerated with increasing oxygen and pH levels. Opiate analogues possessing a free phenolic group at the C-3, like morphine and 6AM, have been shown to undergo microbial oxidation (Stabler and Bruce 1998). The authors did not characterise the microbial oxidation product of 6AM but reported that for morphine this reaction resulted in the formation of pseudomorphine.

Morphine and its glucuronides have been shown to be stable in unpreserved fresh blood and plasma following storage in the dark for six months at 4°C and -20°C (Skopp et al. 2001b). Following 6 months dark storage at 20°C the concentration of morphine and M6G in plasma had declined by 23% and 14% respectively. In whole blood respective losses of 6% and 23% were observed indicating that morphine has greater stability in whole blood compared to plasma. Under the same conditions M3G was stable in both biological matrices. In post-mortem cardiac blood samples however, M3G was found to have significantly greater instability than morphine and M6G, experiencing a concentration decrease of as much as 40%, depending on the sample, when stored at 4°C. The greatest decline in M3G was observed in the sample which had the longest time span between death and collection. The stability of morphine, M3G and M6G in plasma was reduced substantially when samples were stored in light. The same instability was not observed in fresh or post-mortem whole blood (Skopp et al. 2001b).

In highly putrefied blood samples, hydrolysis of morphine glucuronides may occur during sample storage due to residual glucuronidase (GCR) activity following post-mortem bacterial invasion. The residual activity of endogenous GCR over time has been demonstrated in serum and plasma (Lombardo et al. 1980). GCR still had approximately 100% activity in serum and plasma following storage at -20°C and 4°C for 20 and 2 days respectively. The post-mortem stability of this enzyme may account for increases in the concentration of free morphine observed in blood and tissues *in vitro* (Moriya and Hashimoto 1997; Carroll et al. 2000; Skopp et al. 2001b) and highlights the importance of preserving post-mortem blood samples to inhibit bacterial growth. Spiehler and Brown (1987) found the ratio of free morphine to total morphine to be stable in post-mortem blood preserved with 1% NaF and potassium oxalate after more than a year of storage at room temperature. However, even with the addition of sodium fluoride, hydrolytic activity may persist for months (Carroll et al. 2000).

The stability of 6AM in tissues and fluids constitutes a major gap in the published literature. Where information is available, it is based on small sample numbers and limited study conditions. To date there is no data pertaining to the stability of 6AM in muscle tissue. Further investigations in this area are thus required.

2.8.2 Cocaine and metabolites

The stability of cocaine and its metabolites has been well characterised in blood, plasma (Stewart et al. 1977; Liu et al. 1982; Baselt 1983; Garrett and Seyda 1983; Matsubara et al. 1984; Inaba 1989; Isenschmid et al. 1989; Brogan et al. 1992; Klingmann et al. 2001; Skopp et al. 2001a) and urine (Vasiliades 1993; Hippenstiel and Gerson 1994; Levine et al. 1996; Kiszka et al. 2000; Zaitsev et al. 2007), whereas its stability in VH has not been previously reported. At physiological pH (pH 7.4) and temperature (37°C) cocaine undergoes spontaneous chemical hydrolysis to form BZE at a rate of 4.8% per hour in buffer, serum, plasma and whole blood (Taylor et al. 1976; Inaba 1989). The rate of chemical hydrolysis is known to increase with increasing storage temperature and pH. The hydrolysis of cocaine in buffer at $\text{pH} \geq 7$ is reported to be significant (Gupta 1982; Isenschmid et al. 1989; Kiszka et al. 2000; Romolo et al. 2003). Chemical hydrolysis could be slowed with acidification to pH 5 and with low storage temperatures (Isenschmid et al. 1989; Brogan et al. 1992; Kiszka et al. 2000).

Due to residual butyrylcholinesterase (BuChE) activity in blood the enzyme mediated conversion of cocaine to EME continues during sample storage (Isenschmid et al. 1989; Garrett et al. 1994). The rate of this hydrolysis has also been reported to increase with increasing storage temperature and pH (Stewart et al. 1977; Isenschmid et al. 1989; Skopp et al. 2001a). Enzyme hydrolysis may be inhibited by decreasing or blocking the enzyme activity. This may be achieved with low storage temperatures, sample acidification, and by the addition of cholinesterase inhibitors, such as sodium fluoride (Stewart et al. 1977; Isenschmid et al. 1989; Baselt et al. 1993; Skopp et al. 2001a).

Sodium fluoride has been shown to slow down the enzymatic hydrolysis of cocaine to EME through partial inhibition of BuChE, (Stewart et al. 1977). The activity of BuChE has been shown to be stable for 7 days in plasma samples stored at 4°C and 25°C (Balland et al. 1992). In another study the average decrease in BuChE activity after 37 days at 4°C was reported to be only 4.7% in unpreserved plasma and 41.3% in plasma

preserved with sodium fluoride (Lanks and Sklar 1976). Isenschmid et al. (1989) demonstrated that in unpreserved blood cocaine underwent enzyme mediated hydrolysis to form EME exclusively whereas in blood preserved with NaF, cocaine hydrolysed to form BZE only. The rate of BZE formation in blood was much slower than in buffer at the same pH.

In non-fluoridated blood and plasma containing 1 mg/L cocaine and stored at 4°C, cocaine was no longer detectable after 8 and 21 days respectively (Baselt 1983). With the addition of 0.5% NaF, 80% of the original cocaine concentration was detectable after 21 days in both blood and plasma. At 6 weeks cocaine could not be detected in the fluoridated samples. Fluoride preservation with a final concentration of 1 – 5% sodium fluoride (NaF) by weight is recommended for post-mortem cocaine analyses (Skopp 2004) along with immediate refrigeration and prompt analysis. Even in frozen blood samples the addition of preservative is vital for cocaine analyses. A 25% loss of cocaine in unpreserved samples has been reported following storage at -20°C for 110 days. In samples preserved with 2% NaF no decrease in cocaine concentration was noted under the same conditions (Isenschmid et al. 1989). Cocaine in blood specimens preserved with 2% NaF and adjusted to pH 5 was essentially stable when refrigerated or frozen for a period of 200 days.

Cocaine stability also seems to be dependent on the type of blood utilized in the study. It has been reported that cocaine hydrolyses more rapidly to EME in blood bank blood compared to pooled post-mortem blood (Isenschmid et al. 1989). This observation was attributed to the higher pH of the blood bank blood (pH 7.4) compared to the post-mortem blood (pH 6.8) used in the study. A continued decrease in the pH of post-mortem blood was observed as decomposition progressed, particularly at room temperature, thus enhancing the stability of cocaine compared to blood bank blood.

It has been suggested that initial cocaine concentration influences the rate of enzymatic hydrolysis. At very high initial concentrations (90 - 900mg/L), Stewart et al. (1977) reported there to be no measurable hydrolysis in human plasma. Possible saturation of the enzymatic pathway at high cocaine concentrations was thought to account for this observation (Garrett and Seyda 1983). At concentrations more typically found *in vivo* (0.25 – 1.0 mg/L), the rate of cocaine hydrolysis has been shown to be independent of

initial cocaine concentration (Stewart et al. 1977; Isenschmid et al. 1989; Baselt et al. 1993).

Further hydrolysis of EME and BZE to form ecgonine has also been demonstrated *in vitro* (Skopp et al. 2001a). Chemical and enzyme mediated hydrolysis have been proposed in the conversion of EME to ECG and of BZE to ECG respectively (Stewart et al. 1977; Skopp et al. 2001a). In blood and plasma samples stored at 4 and 20°C, the amount of ECG produced from BZE has been shown to be considerably smaller than that produced from EME (Skopp et al. 2001a). Isenschmid et al (1989) also observed BZE to be considerably more stable in whole blood compared to EME indicating that the spontaneous chemical hydrolysis of EME to ECG occurs to a greater extent in stored blood and plasma compared with the enzyme mediated conversion of BZE to ECG. A potential explanation may relate to the different affinities of BZE and cocaine toward the BuChE enzyme (Smirnow and Logan 1996). Ecgonine itself has been found to be particularly stable in blood and plasma samples, accounting for 80% of the initial cocaine concentration after 3 days of storage at 40°C (Skopp et al. 2001a). This metabolite is suggested to be an important marker for cocaine use even when samples have been stored under unfavourable conditions prior to analysis (Logan 2001; Skopp et al. 2001a).

Whilst the stability of cocaine in aqueous solution and in conventional fluids, i.e. blood, serum, plasma and urine, has been well characterised, there is currently no data on its stability in VH. Information on the stability of cocaine in muscle tissue is limited to a single study in which stability was assessed at room temperature and over a period of only 5 days (Section 2.7.2) (Moriya and Hashimoto 1996). As with 6AM then, further investigations are required to characterise the stability of cocaine in stored VH and muscle tissue.

2.9 Rationale

Given the instability of opioids, cocaine and their respective metabolites in blood during the post-mortem period, skeletal muscle tissue and vitreous humour, which are less affected by bacterial action and change in the early post-mortem period, may provide more stable specimens for toxicological analysis. Providing the relationships between drug concentrations in these tissues and blood at the time of death can be clearly established, their use in the quantitative corroboration of a blood sample or as quantitative specimens in the absence of blood may be possible. In order for skeletal muscle tissue to be of any quantitative value a thorough investigation of the drug distribution within a single muscle must be undertaken. The stability of the drugs and their metabolites in these tissues during the putrefactive process and during sample storage must also be well understood if reliable interpretation of toxicological measurements in these matrices is to be made.

Chapter 3.0 Analytical Methodology

In light of the increasing occurrence of polydrug deaths involving both opiates and cocaine, and given that the available sample volume is usually small in forensic cases, a method for the simultaneous extraction and quantification of cocaine, BZE, COET, morphine, codeine and 6AM in femoral blood, vitreous humour and skeletal muscle tissue was developed. The optimised assay involved solid phase extraction of the analytes from each biological matrix followed by derivatisation and quantification using gas chromatography-ion trap-tandem mass spectrometry (GC-MS/MS).

This chapter discusses the principals and the development of the analytical methods used in the present research. It will begin by giving a description of the ion trap method used to obtain mass spectrometric data and a brief overview of analyte confirmation and quantification. The optimisation of the tandem mass spectrometry assay is also discussed followed by an assessment of instrument performance using the optimised MS/MS assay. Development of sample preparation procedures is discussed thereafter. Full details of the final optimised assay and its validation are described in the final section of this chapter (Section 3.9).

The validated method described in this chapter was used in the determination of drug and metabolite concentrations in all stability and case samples analysed in this study (results presented in Chapter 4, 5, 6 and 7). All of these extractions were carried out by the author with the exception of blood and vitreous humour stability samples (Chapter 4 and 5) which were extracted with the assistance of Nicola Jones. Validation of the analytical method was carried out prior to the analysis of research samples. All validation extractions were undertaken by the author with the exception of intermediate precision samples, which were extracted by Poppy McLaughlin in order to assess the method precision obtained by a different analyst on a different day (Section 3.9.3.3).

3.1 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is an analytical procedure that combines the features of gas-liquid chromatography and mass spectrometry to separate and identify different components within a given sample. The specificity and sensitivity

that can be acquired using GC-MS has seen the technique labelled the ‘gold standard’ for forensic toxicological substance identification.

GC-MS analyses gas-phase ions formed from a sample in terms of their mass-to-charge ratios (m/z) and their relative abundances in the resulting spectra. The mass spectrum is a graphical representation of the ion intensities versus the m/z . Under constant, hard electron ionisation conditions (typically 70eV) a sample molecule will fragment into smaller ions of characteristic and highly reproducible m/z values and relative abundances. This fragmentation pattern is a molecular fingerprint for a given analyte, which can be compared to the mass spectrums of drug standards for ultimate identification. Identification of a compound is also achieved by retention time data. The separation of a mixture of compounds in a sample is based on 1) the affinity of the molecules for the column stationary phase and; 2) the boiling point of the molecule. Molecules having a greater affinity for the stationary phase will spend more time on the column and finally elute when the oven temperature reaches the molecules boiling point. Oven temperature may be gradually increased throughout the analysis so that compounds may be separated and elute in order of increasing boiling point.

GCMS method optimisation was carried out on a BPX5 analytical column (30 m \times 0.25 mm internal diameter \times 0.25 μ m film thickness) with 5% phenyl - 95% methylpolysiloxane stationary phase (SGE, Ringwood, Australia). Ultra high purity helium was used as carrier gas at a flow rate of 1.0 mL/min. GC analyses were performed on a Varian 2200 gas chromatograph equipped with a Saturn 2000 ion-trap detector and 8200 Autosampler (Agilent, Stockport, UK). Transfer line, manifold and trap temperatures were set at 260, 100 and 200°C respectively as per manufacturer recommendations. The ion-trap analyser differs from beam transport analysers such as magnetic sector, quadrupole and time-of-flight instruments where the ions to be separated pass through a sequence of fixed electromagnetic fields. Instead, the ion-trap confines the ions within a single region where they experience time-dependent electromagnetic fields.

3.1.1 Ion-trap mass spectrometry

The ion trap can be operated in the full mass scanning mode or by using ion preparation methods, such as tandem mass spectrometry (MS/MS). In full mass scanning mode all

analytes present in a sample are scanned simultaneously. This may be problematic when analysing post-mortem biological samples because it is often difficult to purify the sample sufficiently so that ions from endogenous compounds do not interfere with the identification and quantification of the analytes of interest. The resulting mass spectrum for any particular analyte may contain many additional ions that can mask the true mass spectrum of the target analyte. This was found to be a problem in the initial stage of method development when using full mass scanning. One of the main advantages of the ion-trap is that when further sensitivity and selectivity is required, ion-preparation methods, such as MS/MS, can be used without the need for additional analysers as would be required using a quadrupole instrument. MS/MS effectively removes matrix interference by isolating a single precursor ion from which a full product ion spectrum can be produced by collision induced dissociation (CID). The result is a single analyte peak with minimal interference that can be integrated and quantified.

There are four basic operations in MS/MS for electron ionisation (EI):

- i. *Ion formation and matrix ion ejection:* Ionisation of the sample and any co-eluting matrix occurs by electron ionisation.
- ii. *Parent ion isolation:* During ionisation, a broadband multi-frequency waveform is applied to the end caps of the ion trap to resonantly eject ions below the specified parent ion mass. Following ionisation, a second broadband waveform is applied to the trap to eject those ions whose masses lie above the specified parent ion mass
- iii. *Product ion formation:* Product ions are formed from the isolated parent ion by collision-induced dissociation (CID).
- iv. *Product ion mass scanning:* The product ion spectrum provides qualitative structural information about the sample.

In MS/MS analysis precursor ion isolation, CID and product ion scanning occurs within a pre-defined detection window, or MS/MS segment. Within this segment a single target peak is produced which allows integration and measurement of the chosen compound with minimal matrix interferences. By eliminating interfering matrix ions during parent ion isolation this ion preparation method increases the signal-to-noise ratio. The ability of ion trap to simultaneously monitor all ions of interest means that a complete product ion spectrum is retained. To achieve a similar level of sensitivity with

a quadrupole instrument, the RF is set to select only ions with a particular m/z value (single ion monitoring or SIM). SIM allows for a longer time to be spent monitoring a single ion and for rapid switching between other selected ions resulting in increased sensitivity towards the selected analytes and reduced noise. However, the overall amount of MS data obtained with SIM is reduced because only a few selected ions are detected. Compared to SIM there is no loss of data with ion-trap MS/MS and comparison of the unknown to a reference standard can utilize the whole spectrum as opposed to only a few ions.

3.2 Analyte identification and confirmation

Analyte identification in full scan mode and in SIM using quadrupole instruments is achieved by comparison of retention times and by comparison of the relative abundance of major fragment ions to those values obtained for standards assayed in the same run. Typically three ions are monitored and utilised in the confirmation of compound identity. When using MS/MS the option of using ion ratios to confirm the identity of a compound is available, however, by choosing to use only three ions when the whole product ion spectrum is available means that valuable data is effectively being discarded. As a result of matrix interferences, ion ratios are necessary for analyte confirmation in full scan analysis. They are also necessary in SIM with quadrupole instruments because only a few ions are monitored at one time and thus a full MS profile is not available. For the MS/MS assay compound identification was achieved by use of the full product ion spectrum. Confirmation was achieved when the MS spectrum matched that of concurrently run standards at a match threshold greater than or equal to 900 (90% match) and retention time data was $\pm 1\%$ that of the reference standards.

3.3 Analyte quantification

Analyte quantification was achieved by means of an internal standard calibration graph plotting analyte response divided by IS response versus concentration in calibration standards. Because of the degree of ionisation in MS and the potential for drug loss during sample preparation, the use of an internal standard (IS) is vital when performing quantitative measurements. Even with analyte losses during sample preparation the ratio of the sample to the IS will remain constant and the measured concentration should

reflect more accurately that of the original biological sample. The concentration of an analyte in an unknown sample may be calculated against the corresponding calibration curve providing the detector response is proportional to analyte concentration in the calibration standards.

Deuterated internal standards were chosen for use in the current assay. Stable isotope-labelled analogues, particularly deuterated analogues, are the most commonly used IS's as they have almost identical chemical and physical properties to the analyte and thus extraction, derivatisation, chromatography and fragmentation are virtually identical. Deuterated analogues of COET and codeine were not utilised because the structural similarity between COET and cocaine, and between codeine and morphine, meant that acceptable accuracy and precision could be obtained using cocaine-d3 to quantify COET and morphine-d3 for codeine (Section 3.9). The IS concentration was chosen as the concentration giving a peak response that was approximately equal to the response obtained from the non-deuterated analogue at the mid-point of the calibration range.

3.4 Optimisation of the GC-MS/MS assay

Several software and dissociation parameters had to be optimised so that suitable MS/MS product ion spectra could be obtained for each analyte. A description of each of these parameters and the steps taken for optimisation are given in Appendix 1. Prior to MS/MS method development the oven temperature programme was optimised to obtain the best analyte separation possible within an acceptable run time (Figure 4). To enable CID to produce a product ion spectral profile in which the precursor ion represented the base peak and the major product ions ranged between 40 - 100% the abundance of the base peak, the voltage applied to the precursor ion (excitation amplitude) for CID was optimised. This was achieved using automatic method development (AMD), an automated feature of the Saturn 2000 MS software. AMD enables several voltages to be applied simultaneously to the isolated ion speeding up the optimisation process. Under electron impact conditions the parent ions of cocaine, COET and BZE were not well retained. More stable product ions were selected for use as precursor ions (Table 5).

Because of the chemical similarity between an analyte and its deuterated analogue, elution of these compounds from the analytical column occurs simultaneously. To

achieve simultaneous scanning of deuterated standards and their non-deuterated analogues within a single MS/MS segment the ion trap was operated in multiple reaction monitoring (MRM) mode. In MRM two separate precursor ions can be isolated and subjected to CID, each in a separate scan channel but within the same time segment. Despite extensive development of the oven temperature program, COET and BZE co-eluted. In this instance multiple reaction monitoring was utilized for three compounds; COET, BZE and BZE-d3. In scanning multiple analytes in a single time segment reduction in the mass resolution is a possibility. Nevertheless, by reducing the scan time for this segment, it was possible to scan all three compounds simultaneously with no visible reduction in mass resolution. The optimised CID spectra for each analyte are displayed in Figures 5 and 6. Suggested dissociation pathways forming the major product ions, and in the case of cocaine and its metabolites, the fragmentation pathway also forming the precursor ions, are displayed in Figures 7 and 8. Optimised collision induced dissociation (CID) and compound specific parameters are reported in Table 5. Quantification ions and internal standard allocations are also reported. The GC-MS/MS method is described in full in the validation section (Section 3.9.2.8).

3.5 Derivatisation

Derivatisation is often necessary to achieve satisfactory chromatography and to improve detection of an analyte. Compounds containing hydroxyl groups are not usually amenable to GC because of their polarity. Four of the six analytes studied (BZE, codeine, morphine and 6AM) contain hydroxyl groups and thus required derivatisation. The most commonly used derivatisation method for opiates, cocaine, and their respective metabolites is via silylation, where OH groups are replaced by a trimethylsilyl (TMS) group. Silylation can be achieved by addition of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), either alone or with 1% trimethylchlorosilane (TMCS) added as catalyst, or *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), each followed by heating. The addition of BSTFA+ 1% TMCS followed by heating at 70°C for 20 minutes is commonly used for the drugs of interest (Mackey-Bojack et al. 2000; Wyman and Bultman 2004; Antonides et al. 2007) and thus was chosen for use in the current method.

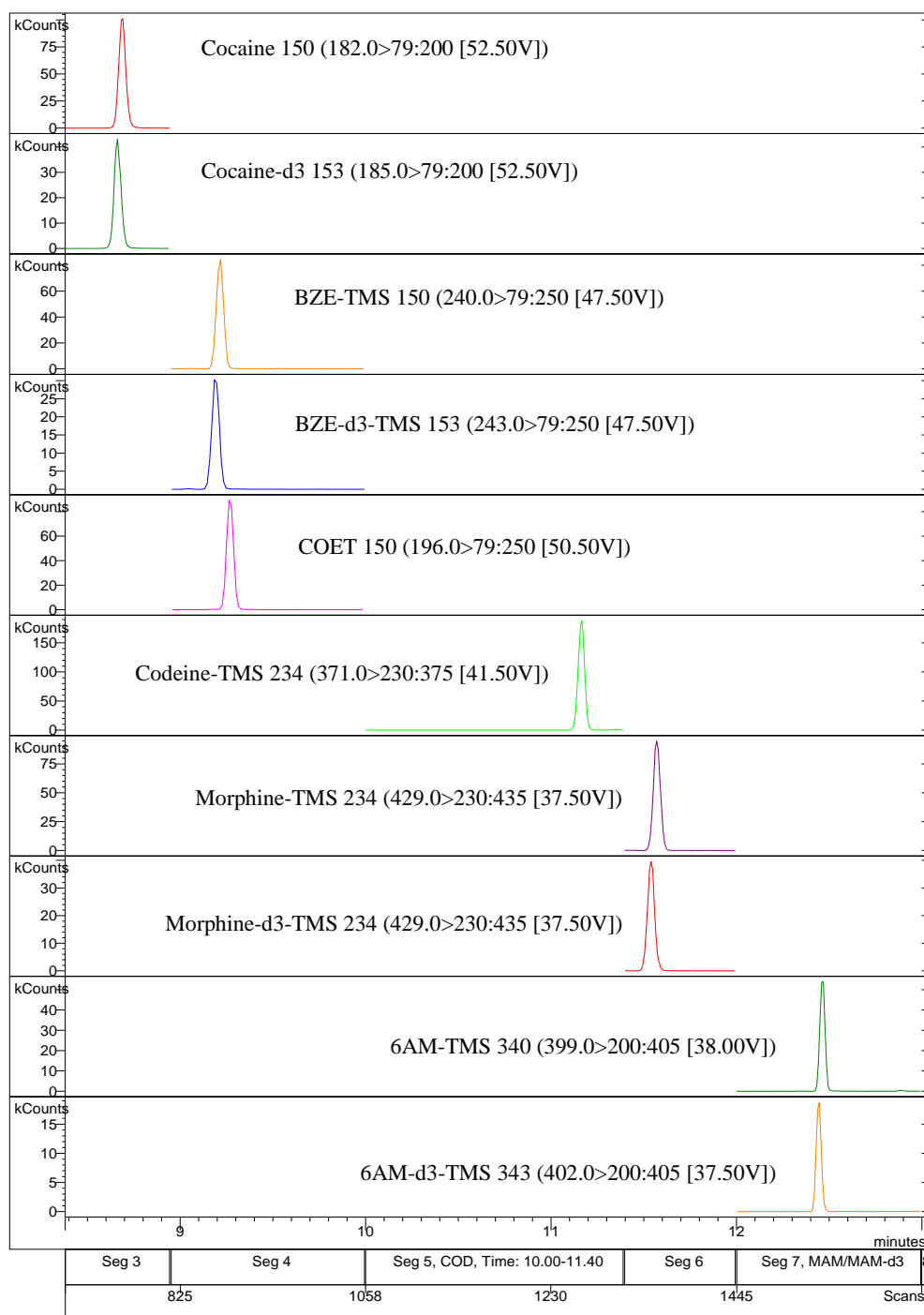
It is known that the use of derivatisation reagents can rapidly degrade the analytical column and so initial experiments using morphine involved drying down the derivatising reagent following heating and then reconstituting in solvent. However, using this method an additional peak consistently eluted just before the morphine peak (Figure 9). Experiments were conducted to rule out contamination as a potential source. It was noted that a longer heating time reduced the size of the peak and allowing overnight derivatisation removed it completely which suggested it to be an artefact of incomplete derivatisation. On analysis of the fragmentation pattern of the erroneous peak, it was discovered that it was mono-derivatised morphine. The mono-derivative was not observed when the standard was injected with the derivatisation reagent, indicating that the derivatisation occurs most efficiently in the hot injector port (maintained at 260°C). The importance of injecting the sample with the derivatisation reagent was clear from these results. In experiments comparing the use of 25µL, 50µL and 100µL derivatising reagent, no difference in peak response was observed. In order to preserve the column the smallest amount of reagent (25µL) was used in subsequent experiments with an additional 25µL of ethyl acetate to give a final sample volume of 50µL.

To optimise the derivatisation procedure and to assess the short-term stability of the derivatised products, the effect of post-derivatisation standing time on peak response was investigated. In order to obtain the best limits of detection and quantification the standing time giving the best peak response was considered advantageous. Derivatised samples were allowed to stand at room temperature for 0, 2, 6 and 24 hours. Following instrument failure it is necessary for processed samples to be stored for a period until maintenance can be performed. For this reason the stability of samples having stood for 24 hours at room temperature was further assessed following 48 hours refrigerated storage (74 hours total). The results are displayed as relative response (%) for each of the conditions studied (Table 6).

Table 5 Collision induced dissociation parameters, compound specific MS/MS parameters, and details of quantification in the MS/MS assay

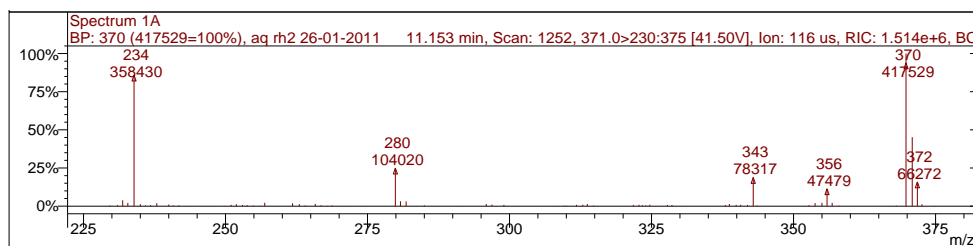
Compound (IS allocation)	Segment time (min)	Retention time (min)	Precursor ion (m/z)	Scan time (s)	Mass range (m/z)	Excitation amplitude (v)	Total ion current (counts)	Quantification ion (m/z)
Cocaine (cocaine-d3)	8.00 – 9.0	8.711	182	0.28	79 – 200	52.5	10,000	150
Cocaine-d3		8.682	185			52.5		153
COET (cocaine-d3)	9.0 – 10.00	9.292	196	0.28	79 – 250	50.5	10,000	150
BZE-TMS (BZE-d3)		9.240	240			48.0		150
BZE-TMS-d3		9.204	243			47.5		153
Codeine-TMS (morphine-TMS-d3)	10.0 – 11.4	11.192	371	0.38	230 – 375	41.5	5,000	234
Morphine-TMS (morphine-TMS-d3)	11.4 – 12.0	11.603	429	0.28	230 – 435	37.5	5,000	234
Morphine-TMS-d3		11.561	432			37.0		237
6AM-TMS	12.0 – 13.0	12.473	399	0.28	200 – 405	38.0	5,000	340
6AM-TMS-d3		12.455	402			37.5		343

TMS: trimethylsilyl derivatised product

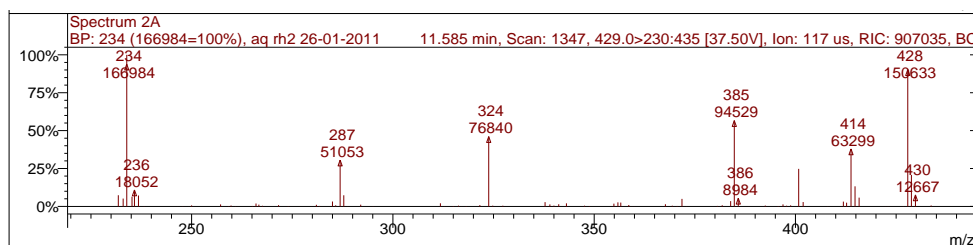


The quantification ion is stated after the compound name. In brackets: precursor ion; mass range; [excitation amplitude, V].

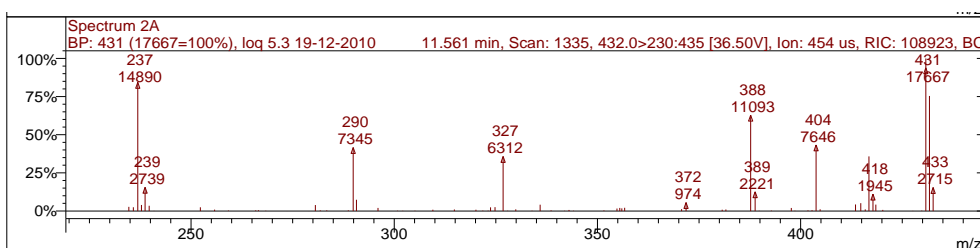
Figure 4 Chromatograms of cocaine, cocaine-d3 (segment 3), BZE-TMS, BZE-d3-TMS and COET (segment 4), codeine-TMS (segment 5), morphine-TMS, morphine-d3-TMS (segment 6), 6AM-TMS and 6AM-d3-TMS (segment 7)



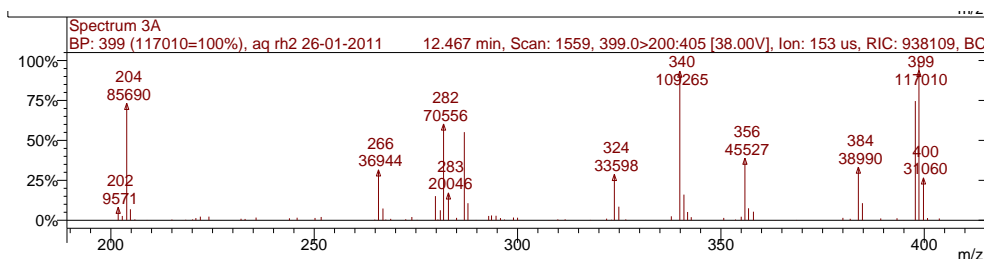
(A)



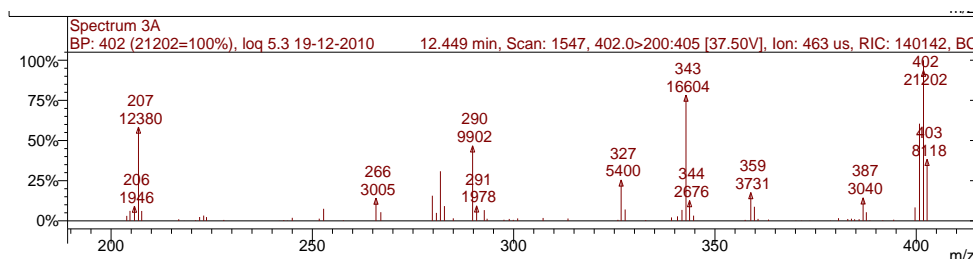
(B)



(C)



(D)



(E)

Figure 5 Collision induced dissociation spectra of codeine (A), morphine-TMS (B), morphine-d₃-TMS (C), 6AM-TMS (D) and 6AM-d₃-TMS (E) obtained in electron impact mode with MS/MS ion preparation.

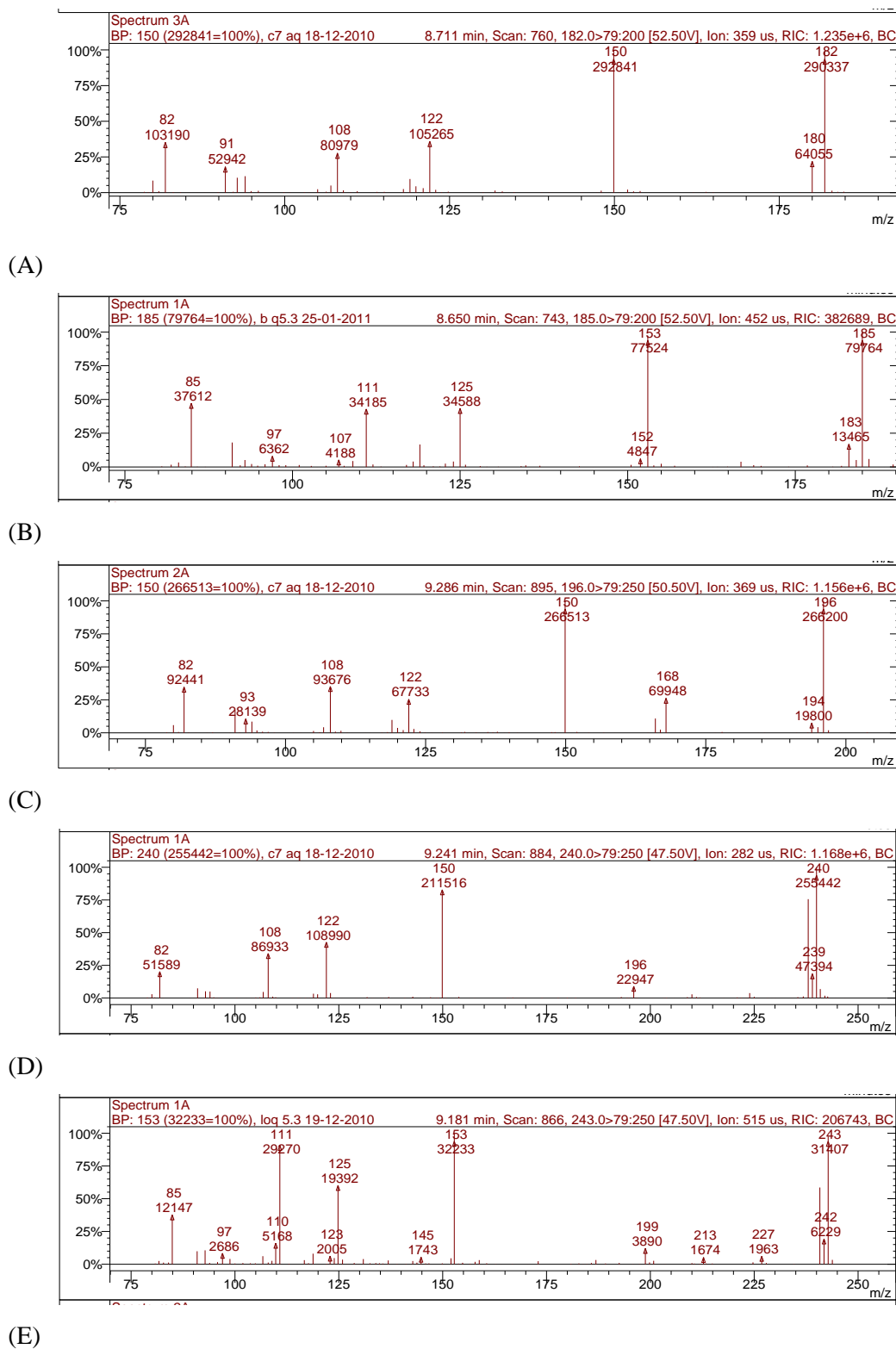


Figure 6 Collision induced dissociation spectra of cocaine (A), cocaine-d3 (B), COET (C) BZE-TMS (D), BZE-d3-TMS (E) obtained in electron impact mode with MS/MS ion preparation.

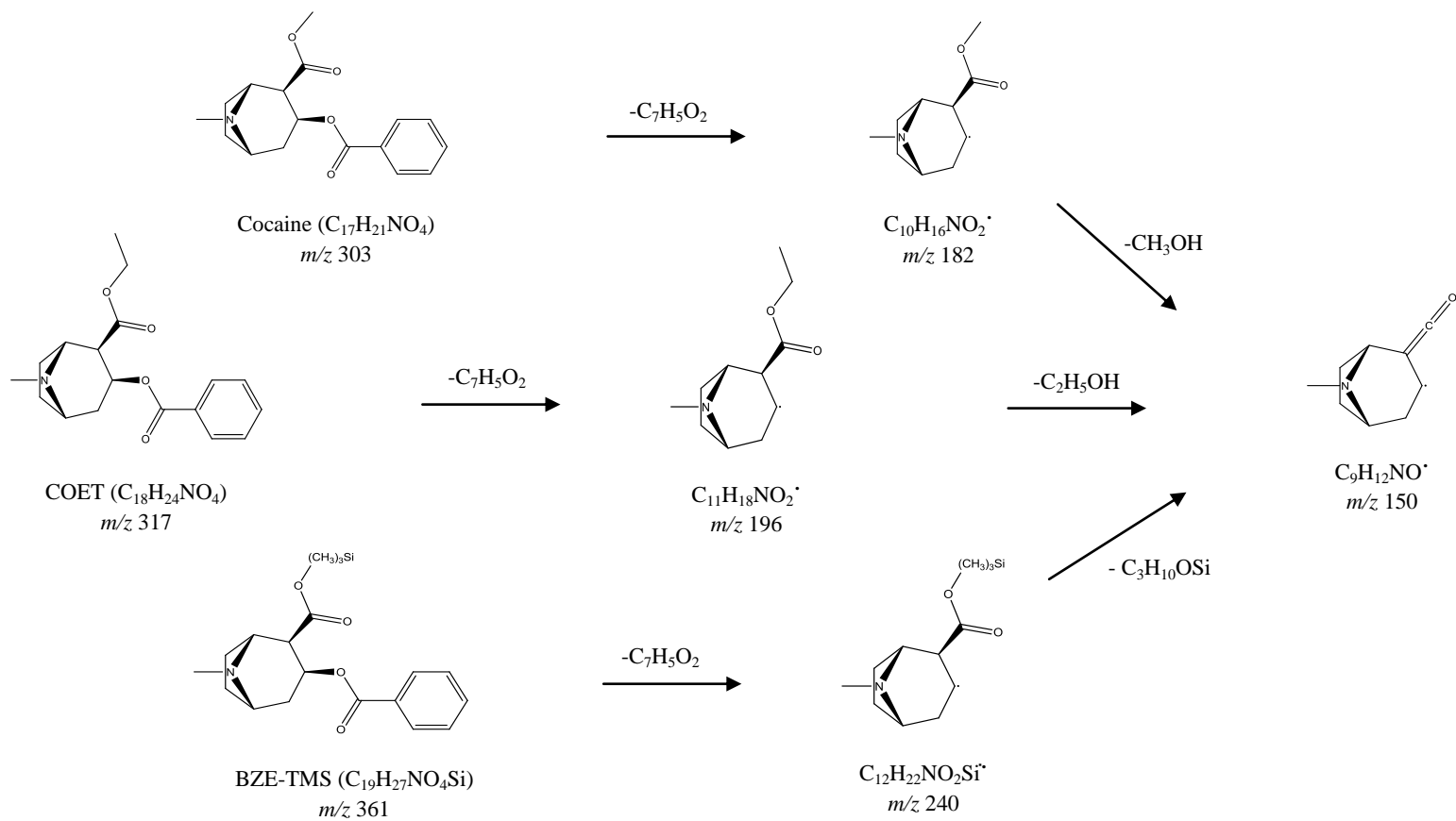


Figure 7 Proposed mass fragmentation of cocaine, COET and BZE-TMS.

Pathways demonstrate the formation of precursor ions; m/z 182, 196 and 240 for cocaine, COET and BZE respectively, and further fragmentation to form the common quantitative ion (m/z 150).

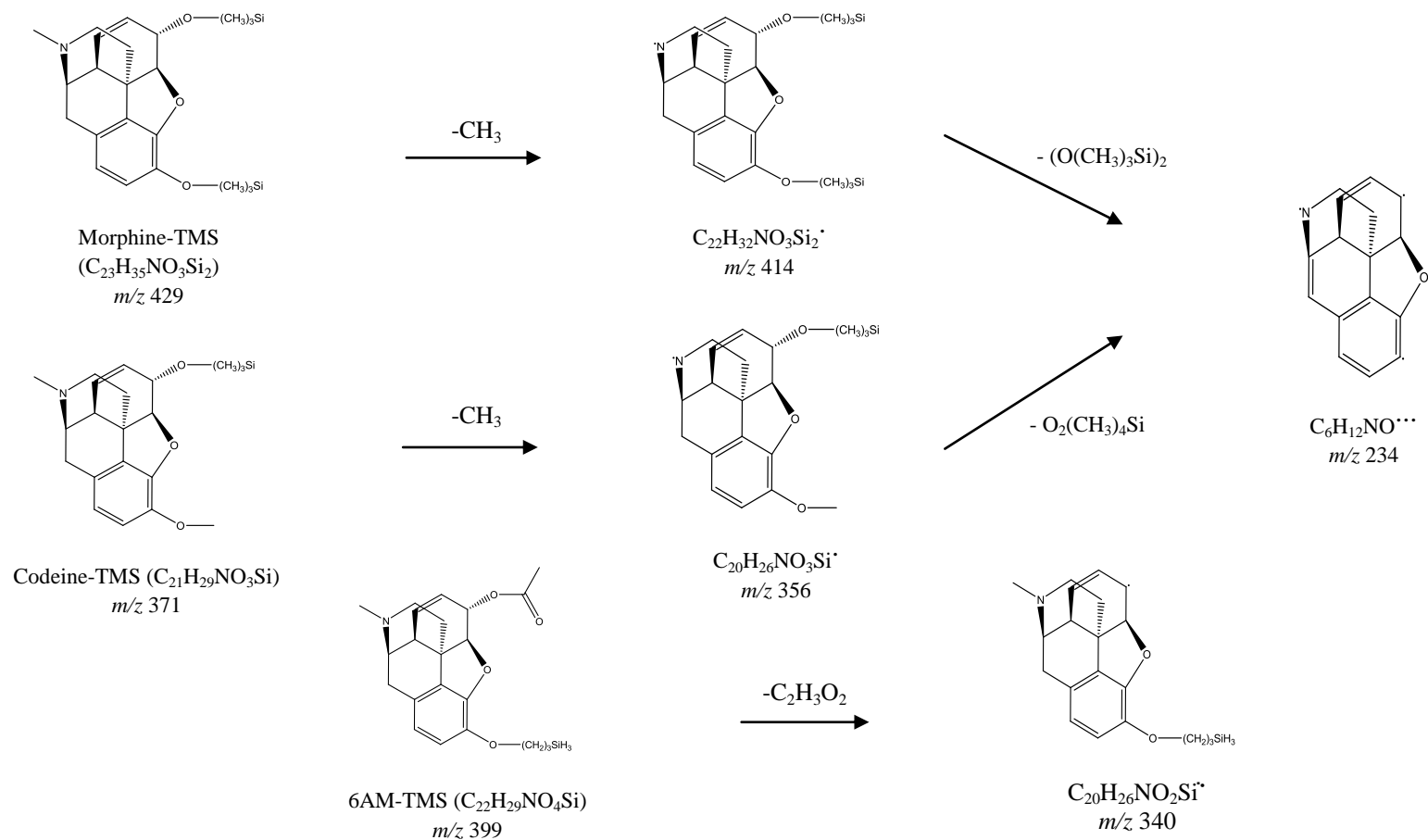


Figure 8 Proposed mass fragmentation of morphine-TMS, codeine-TMS and 6AM-TMS.

Pathways demonstrate the formation of quantitative ions for morphine (m/z 234), codeine (m/z 234) and 6AM (m/z 340).

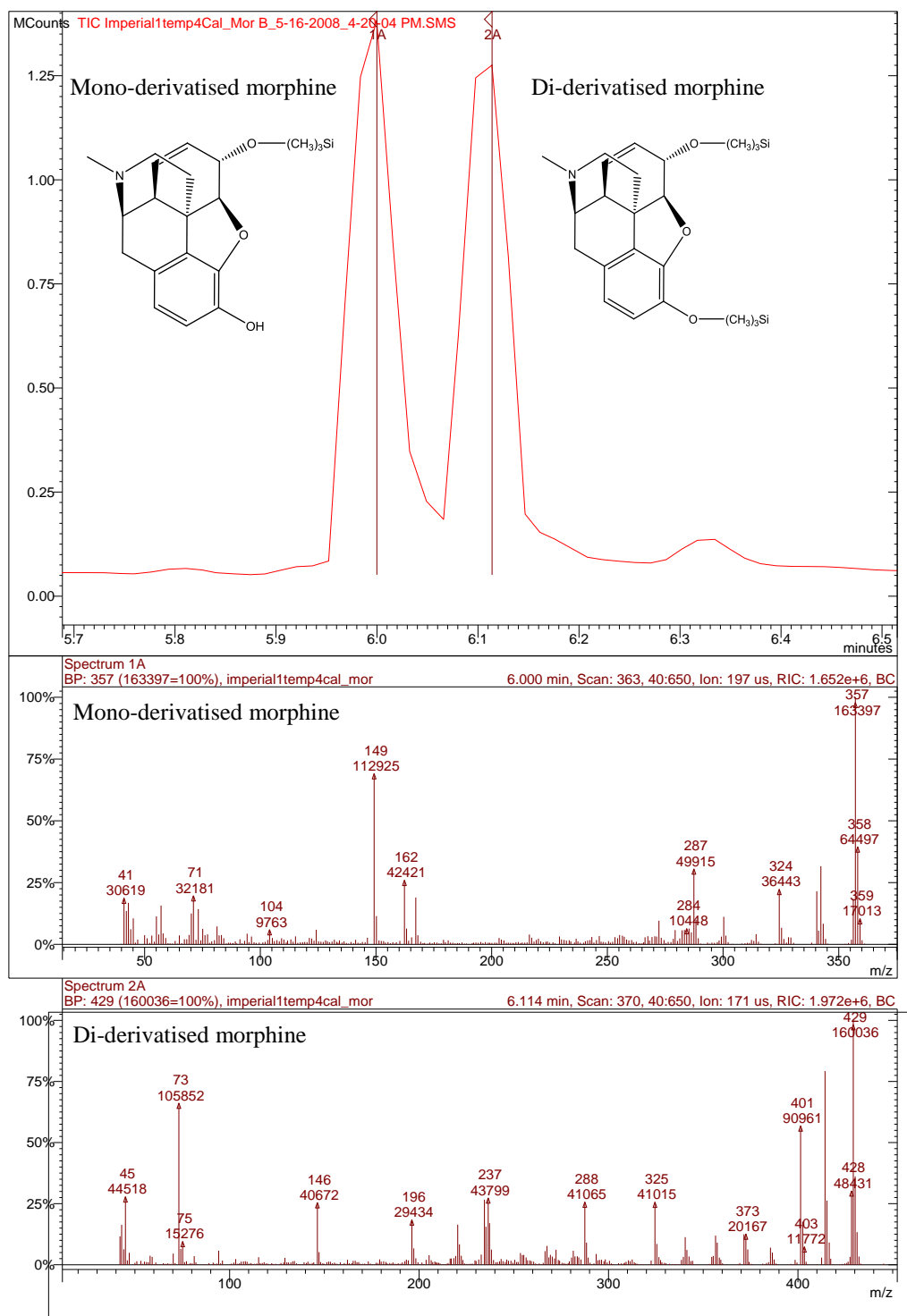


Figure 9 Duplicate morphine peaks with different mass spectral profiles resulting from complete (di-TMS) and incomplete (mono-TMS) derivatisation of morphine

Table 6 Effect of post-derivatisation standing time on relative peak response

Standing time (hours)	Relative response (%)					
	Cocaine	COET	BZE	Codeine	Morphine	6AM
0 (ND)	51	55	54	54	56	62
0	75	78	70	76	76	92
2	91	93	89	94	93	100
6	100	100	100	100	100	95
24	74	75	89	76	77	60
74*	58	71	70	43	46	35

ND = non-derivatised (no heat applied following addition of BSTFA+TMCS)

74* = 24 hours standing at room temperature and 48 hours stored at 4°C

Although 6 hour standing gave slightly better response than 2 hours, the difference was not considered significant and a shorter overall analysis time was preferable. Thus, it was decided that samples would be allowed to stand for a minimum of 2 hours prior to sample injection. There was a clear reduction in peak response following the 74 hour delay, nevertheless, samples quantified accurately with internal standard calibration. On several occasions during real case analyses and during method validation, samples had to be stored for periods ranging from 1 – 10 days whilst the instrument underwent maintenance. In each case quality control and validation samples were quantified accurately and with good precision, thus highlighting the importance of internal standard addition.

3.6 Assessing GC-MS method and instrument performance

As a pre-requisite to the assessment of the full assay characteristics, the performance of the GC-MS/MS in the absence of sample preparation was assessed. An instrument assessment procedure was designed to ensure that any error observed in results obtained from samples which had gone through the full preparation procedure would have origin in sample preparation and not be a result of the GC-MS/MS method and/or instrument imprecision.

The parameters assessed were linearity, injector precision and accuracy, limit of quantification (LOQ) and accuracy and precision at the LOQ. To assess instrument performance, calibration standards and test samples were prepared in methanol. Standards were spiked into 900µL methanol to achieve the required concentration. Internal standard was added and samples were taken to dryness at 40°C under a gentle stream of nitrogen then derivatised. Details of the derivatisation procedure and GC-MS/MS method are also given in the validation section (Section 3.9)

3.6.1 Linearity

The linearity of an analytical procedure is its ability, within a given range, to obtain test results which are directly proportional to the concentration of analyte in the sample. Ideally the calibration standard concentrations are chosen so that they bracket the anticipated concentration of the specimen(s), but linearity at the extremes of the concentration range must first be established. Typically, at least three calibrators should be used to establish linearity, although a minimum of five is preferred (Cooper et al. 2010). For a multi-point calibration the criterion for acceptance is usually the correlation coefficient (r); a perfect straight line having a value of 1 (unity). However, a high value of r (approaching unity) does not itself prove that the curve is linear. Simple linear regression (unweighted) treats all points equally as it assumes equal variance at all concentrations (homoscedastic). For calibration data the variance generally increases with concentration (errors are approximately proportional to concentration). In order to reduce heteroscedasticity, linearity was determined using least squares regression with $1/x^2$ ($1/(\text{concentration})^2$) weighting.

Eight concentration levels ranging from 0.005 to 0.2 mg/L for 6AM and 0.01 – 2.0 mg/L for all other analytes was used to establish the instrument limit of linearity in three separate experiments. The limit of linearity was defined as the highest concentration level at which the weighted correlation coefficient squared (coefficient of determination, R^2) for the curve was ≥ 0.99 . BZE and 6AM demonstrated excellent linearity ($R^2 \geq 0.999$) over the full concentration range in each of the three experiments. Cocaine, COET and codeine demonstrated good linearity ($R^2 \geq 0.99$) over the entire range in two out of the three experiments. All three compounds demonstrated excellent linearity up to 1 mg/L ($R^2 \geq 0.999$) in each experiment. Morphine was linear up to 1 mg/L ($R^2 \geq 0.99$). The linearity demonstrated here and the concentrations anticipated in

actual casework were taken into consideration when choosing the final assay concentration ranges for each compound. The final range for 6AM was 0.005 – 0.2 mg/L and 0.01 – 1.0 mg/L for all other analytes (Figure 10).

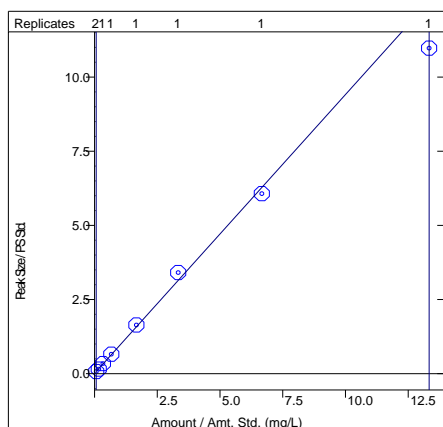
3.6.2 Injector precision and accuracy

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions (Hubert et al. 2004). In assessing instrument performance, the injector precision was determined. This refers to the closeness of agreement between a series of measurements made from multiple injections of the same sample. In precision experiments the percentage relative standard deviation (%RSD) of an assay should not exceed 20% at low concentration (at or near the limit of quantification (LOQ)) and 15% at higher concentrations (Flanagan et al. 2007). The %RSD is typically quoted because the errors are generally proportional to the concentrations being measured. The accuracy, or trueness, of an analytical procedure expresses the closeness of agreement between an accepted reference value and test result (Flanagan et al. 2007). Deviation from the nominal value should not exceed 20% (Cooper et al. 2010)

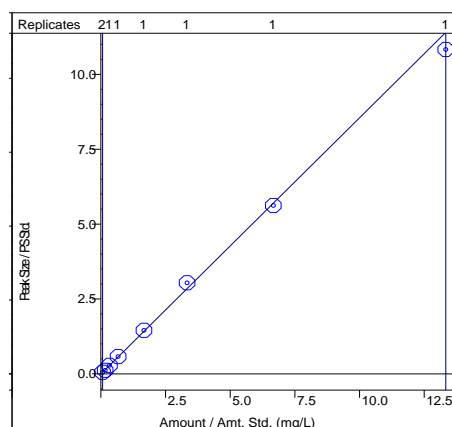
Injector precision and accuracy was determined by preparing single samples at low, medium and high concentration; 0.01, 0.05 and 0.1 mg/L for 6AM and 0.025, 0.15 and 0.4 mg/L for all other compounds. Each test sample was injected eight times. The injector precision was calculated as % RSD of both the peak response and of the eight quantification results. As only one sample per concentration is employed here the precision is related solely to the GCMS method and not to the precision associated with the preparation of the sample. The accuracy was calculated as the percentage deviation of the mean result from the nominal concentration. Injector precision and accuracy results are displayed in Table 7. The injector precision at low, medium and high concentration was $\leq 10\%$ for all compounds. Percentage deviation from the nominal (accuracy) was $\leq 15\%$ for all compounds at each of the three concentrations. Although for most of the compounds the deviation was greatest at the low concentration, no significant trend was observed in the precision and/or accuracy over the three concentrations tested.

Cocaine

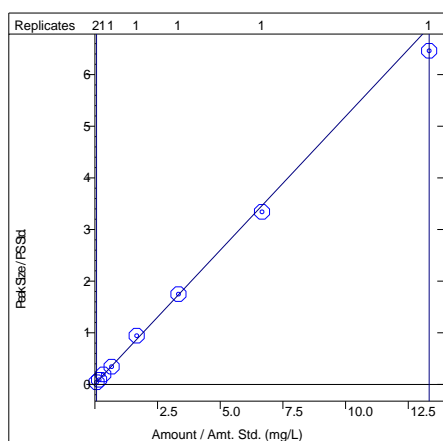
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 8.788%, Coeff. Det.(r2): 0.995277
 $y = +0.9400x + 0.0105$

**Cocaethylene**

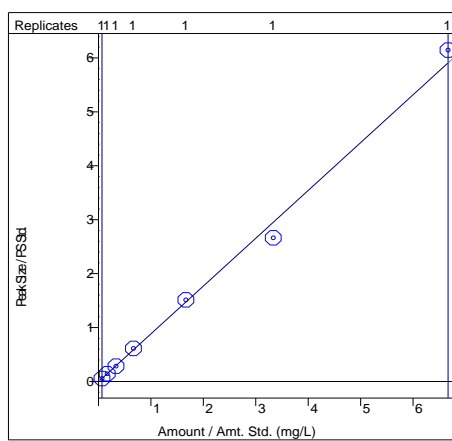
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 6.931%, Coeff. Det.(r2): 0.998928
 $y = +0.8555x - 0.0054$

**BZE**

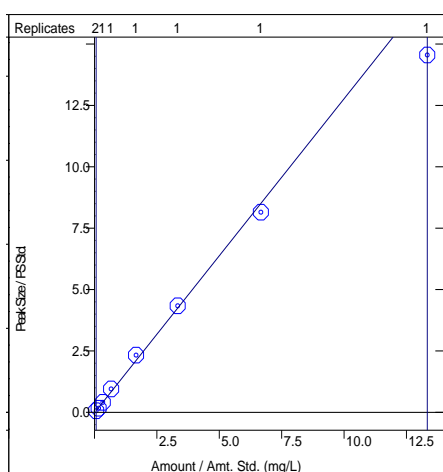
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 9.515%, Coeff. Det.(r2): 0.999300
 $y = +0.5182x + 0.0084$

**Morphine**

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 5.550%, Coeff. Det.(r2): 0.995590
 $y = +0.8867x - 0.0040$

**Codeine**

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 13.28%, Coeff. Det.(r2): 0.995241
 $y = +1.2790x - 0.0196$

**6MAM**

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 10.98%, Coeff. Det.(r2): 0.998808
 $y = +1.1526x + 0.0029$

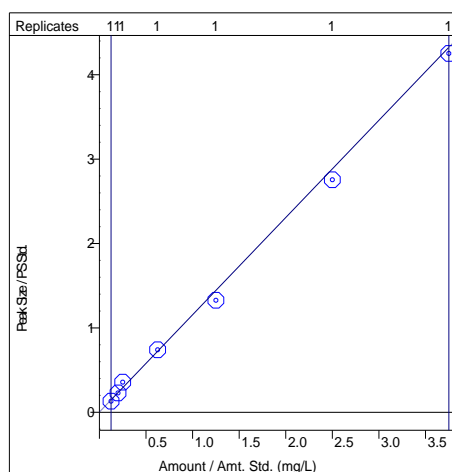


Figure 10 Calibration curves generated in methanolic solution for cocaine, COET, BZE, morphine, codeine (0.01 – 1.0 mg/L) and 6AM (0.005 – 0.2 mg/L)

3.6.3 Instrument limit of quantification

The instrument limit of quantification (ILOQ) was considered a particularly important parameter to assess to ensure that the instrument was capable of reliably quantifying the lowest calibration standards chosen for the final assay (0.005 mg/L for 6AM and 0.01 mg/L for all other analytes). Typically the LOQ is defined as the analyte concentration required to give a peak height ten times that of the background noise level (Cooper et al. 2010). Because the noise is essentially removed in MS/MS this definition was not employed for the current assay. Instead the ILOQ was defined as the lowest concentration for which the %RSD of replicate results was $\leq 20\%$ and the quantification results were all within $\pm 20\%$ of the nominal. Determination of ILOQ was achieved by triplicate injection of standards prepared in triplicate at concentrations of 10, 7.5, 5, 2.5 and 1 ng/mL. The accuracy and precision was calculated as described earlier from the mean of the nine results. The % RSD of the peak response was also calculated although not a determinate factor. Occasionally it was obvious that a single result or peak response was due to injection error where two of the results were in close agreement and one was not, referred to here as a 'flier'. On the few occasions where this was observed, the flier was removed prior to calculation of the mean for that triplicate data set.

Based on the pre-defined criteria the limit of quantification for cocaine, COET, morphine and 6AM was 1 ng/mL and for BZE and codeine the LOQ was 2.5 ng/mL (Table 8). For BZE the accuracy obtained at 1 ng/mL was well within the acceptable 20% deviation (11%), however the precision was marginally greater than the 20% RSD (22%). For codeine the precision was acceptable at 1 ng/mL (11%) but the accuracy was not (33%). The LOQ determined for each compound demonstrated the ability of the instrument to accurately and precisely quantify samples at concentrations well below those chosen for the lowest calibration standards.

3.6.4 Discussion

In assessing the precision over replicate injections at all concentration levels, it was noted that fliers were more common when observing the peak response as compared with the result corrected by internal standard. The %RSD was often higher for the peak response. Considering that this occurrence was observed with samples having undergone minimal sample preparation and, at least for the injector precision

assessment, injected from the same vial, the importance of using an internal standard during extraction was highlighted. Owing to variations in the condition of different biological samples, extraction efficiency may vary quite significantly between samples extracted in the same run. An internal standard is therefore vital to account for losses which may not be consistent across a single run. Owing to the poor reproducibility of peak response but excellent precision of the result when corrected by internal standard, the precision over the peak area was not selected as criteria for acceptance in the full validation procedure (Section 3.9).

Table 7 Injector precision and accuracy

Compound	Nominal Concentration (mg/L)	Mean concentration* (mg/L)	Accuracy (% deviation)	Precision (% RSD result)	Precision (% RSD response)
Cocaine	0.025	0.028	14	6	17
	0.15	0.16	6	4	11
	0.40	0.46	15	4	12
COET	0.025	0.026	3	7	13
	0.15	0.15	2	4	11
	0.40	0.45	13	5	13
BZE	0.025	0.026	5	6	13
	0.15	0.17	12	9	12
	0.40	0.45	11	6	12
Codeine	0.025	0.028	14	7	15
	0.15	0.16	6	7	9
	0.40	0.44	11	8	13
Morphine	0.025	0.028	12	10	9
	0.15	0.16	9	4	9
	0.40	0.43	7	6	13
6AM	0.010	0.011	10	8	19
	0.050	0.052	4	9	9
	0.10	0.099	1	6	15

* Mean of eight repeat injections of a single sample

Table 8 Instrument limit of quantification

Compound	Nominal Concentration (ng/mL)	Mean concentration* (ng/mL)	Accuracy (% deviation)	Precision (% RSD result)	Precision (%RSD response)
Cocaine	10	12	16	6	6
	7.5	7.4	1	9	8
	5	5.7	14	3	18
	2.5	2.7	10	4	11
	1	1.3	0	10	4
COET	10	11	7	2	3
	7.5	8.1	8	5	6
	5	5.9	18	7	10
	2.5	2.6	10	5	11
	1	1.1	8	8	12
BZE	10	10	1	9	4
	7.5	7.6	1	8	14
	5	5.6	12	10	23
	2.5	2.7	7	5	20
	1	1.1	11	22	4
Codeine	10	8.9	11	0	7
	7.5	6.3	16	4	4
	5	4.8	3	3	18
	2.5	2.1	18	10	15
	1	0.7	33	11	8
Morphine	10	11	11	8	3
	7.5	7.0	6	2	5
	5	5.7	14	7	17
	2.5	2.5	7	14	18
	1	0.9	12	2	14
6AM	10	11	9	13	8
	7.5	8.2	10	8	13
	5	5.6	13	7	25
	2.5	2.9	17	6	10
	1	1.0	1	6	7

* Mean of three LOQ samples each injected in triplicate (n = 9)

LOQ for each analyte highlighted in bold

3.7 Sample preparation

The analysis of opiates and cocaine in biological specimens is typically achieved through initial sample pre-treatment by means of either solid phase extraction (SPE) or liquid-liquid extraction (LLE). SPE has been shown to have several benefits over LLE in the extraction of basic drugs (Juhascik and Jenkins 2009), including lower limits of detection, reduction of matrix effects, decreased solvent use, simplified batch processing, increased speed, and capability for automation. For blood and VH, SPE appears to be the standard technique used in toxicology laboratories with the general method typically involving dilution with buffer followed by centrifugation and application of the supernatant to a conditioned SPE cartridge (Ziminski et al. 1984; Logan and Stafford 1990; Mackey-Bojack et al. 2000; Stephen et al. 2006). In developing the present assay this general procedure was found to be simple and effective both for matrices.

For muscle, mechanical homogenisation in buffer and sometimes enzyme digestion is often followed by liquid-liquid extraction (Garriott 1991; Langford et al. 1998; Giroud et al. 2004; De Letter et al. 2007) or more rarely SPE (Klingmann et al. 2000; Cardona et al. 2006; Wyman et al. 2011). Despite the lack of reports on muscle analysis utilising SPE, this extraction technique was chosen for use in the current assay owing to its many advantages over LLE. As with blood and VH, centrifugation of buffer diluted homogenised muscle tissue with subsequent application of the supernatant to SPE proved to be a simple and effective extraction procedure for this matrix. Details of the development of the muscle extraction procedure are given in Section 3.7.4.

3.7.1 Specimens

As a result of the difficulty associated with obtaining authentic human tissue samples, animal equivalents were utilised in the development of the present assay. Oxalated horse blood (TCS Biosciences, Buckingham, UK), sheep VH (Dunbia Abattoir, Carmarthenshire, UK) and butchers meat (lean beef obtained from a local butcher) were chosen owing to their ease of procurement. Horse blood is available commercially and used extensively in toxicology laboratories for preparing matrix-matched blood standards and is believed to be more representative of fresh human blood than blood-bank blood, since the latter is diluted substantially. However, compared with human blood, horse blood contains an additional hydrolytic esterase, carboxylesterase (Aldrich

1953) which is known to hydrolyse ester containing drugs/metabolites such as cocaine and 6AM (Li et al. 2005). For this reason blood calibration standards and controls were prepared fresh daily during the method development process and subsequent validation.

3.7.2 Solid phase extraction

ISOLUTE® Confirm cartridges (10 mL) with 130 mg of HCX (mixed mode) sorbent (Biotage, Uppsala, Sweden) were chosen for use in the current assay. These are co-polymeric ion exchange columns having both hydrophobic functional groups (non-polar chain, C8 group) and ionic exchange functional groups (polar chain, benzene sulfonic acid group). Basic drugs with amine functionalities absorb onto the column via both hydrophobic and ionic attraction. The choice of SPE sorbent was based on the frequently reported use of mixed mode sorbent for the extraction of opiates and cocaine from biological fluids (Goldberger et al. 1994; Broussard et al. 1997; Broussard et al. 2001; Jones et al. 2002; Romolo et al. 2003; Wyman and Bultman 2004; Lewis et al. 2005).

Prior to sample application, the HCX column was conditioned using methanol and 0.1 M phosphate buffer (pH 6.0). Methanol was applied to the column as a wetting agent to expand the functional binding sites away from the sorbent surface and expose them to the diffusive flow of the sample and reagents. Phosphate buffer (pH 6.0) was applied to the column to chemically prepare the sorbent environment allowing optimal contact and binding of the sample, which was also adjusted to pH 6.0 by addition of 0.1M phosphate buffer; the pH of the sorbent is made equivalent to the sample to facilitate mass transfer between the matrix and the sorbent for optimal binding. pH 6.0 was chosen because this is 2 pH units below the lowest pKa of the analytes of interest (morphine pKa = 8.0) and thus ensures that all analytes are at least 99% ionised. At this pH the amine groups will be positively charged (cationic) and absorb onto the column via both ionic attraction and hydrophobic interaction. The benzene sulfonic acid group on the HCX sorbent is anionic (negatively charged) and attracts the positively charged basic drugs (cationic) through cation-exchange forming strong bonds.

Initially 3 mL cartridges were employed but recovery was found to be improved with a greater volume dilution (from 3 mL to 8 mL buffer dilution) so 6 mL volume cartridges were employed. In opiate extractions reported in the literature, the sample and column

may also be adjusted to pH 4.5 with 0.1 M acetate buffer (Telepchak et al. 2004). In recovery experiments comparing the use of phosphate buffer and acetate buffer, phosphate buffer gave consistently better results, particularly for morphine and 6AM where recovery from blood was 20 - 30% greater at pH 6.0.

Wash stages involved the sequential addition of 1) water - to remove polar interferences; 2) dilute hydrochloric acid - to complete the removal of polar interferences and to ensure that the basic analytes remain positively charged, and; 3) methanol - to remove non-polar, non-basic drugs and interferences. Following the acid wash step the cartridges were dried under full vacuum to ensure removal of residual water prior to the organic wash - the presence of water during the organic wash negatively affects the efficiency of the wash by minimising contact of the sorbent with the organic solvent.

The HCX uses a strong ion-exchanger which means it is charged across the entire pH range. Consequently, the only neutralisation that can be done to release the analytes of interest is that of the analytes. A basic elution solvent, dichloromethane / isopropanol / ammonium hydroxide (80/18/3 v/v/v), was used to neutralise the analytes. This solvent should have a pH of 11 - 12, which is at least 2 pH units above the highest pKa of the analytes of interest (BZE pKa = 9) and so ensures that 99% or more neutralisation takes place and the ionic interaction is effectively disrupted so that the analytes are eluted. Typically the proportion of the ammonium hydroxide NH₄OH used in published methods is 2%, however, during the method development for the current assay the recovery was improved with additional NH₄OH (3%). Prior to the elution step the column was dried under full vacuum to remove methanol and any residual moisture; the presence of moisture would prevent the water immiscible elution solvent from optimally interacting with the analytes. The full SPE procedure is described in the validation section (Section 3.9).

3.7.3 Skeletal muscle

Conventionally, drug measurements in muscle tissue have been performed via mechanical homogenisation and/or acid digestion prior to solvent extraction (Garriott 1991; Moriya and Hashimoto 1996; Langford et al. 1998; Giroud et al. 2004; De Letter et al. 2007). Using a conventional extraction technique, only a percentage of the total

drug present in the tissue may be extracted as some will inevitably remain bound in the tissue. Digestion of tissue with enzymes prior to solvent extraction has been shown to give much better recovery compared to direct solvent extraction and acid digestion and without the chemical breakdown associated with the latter (Osselton 1987). Acid digestion was not considered for use in the current assay because 1) acid will deconjugate opiate glucuronides and; 2) 6AM is not stable under acidic conditions (Romolo et al. 2004). Whilst digestion by Subtilisin® Carlsberg has been demonstrated to be effective in the extraction of basic drugs (Osselton, 1977; Hammond and Moffat, 1982; Osselton, 1987, Flanagan et al. 2003; Duer et al. 2006) the optimum pH condition for this enzyme has been determined to be between pH 8.0 and 10 (Osselton, 1977). Exposing samples to any pH within this range at the temperature and duration required for digestion (55°C for one hour) would present problems for the analysis of cocaine and 6AM, both of which are known to break down under alkaline conditions even at room temperature (Isenschmid et al. 1989; Barrett et al. 1992).

In an attempt to develop an enzyme digestion method for muscle tissue analysis, the use of proteinase K from *Tritirachium album* (EC 3.4.21.64; Cat# P8044, Sigma-Aldrich, Poole, UK) was investigated. Proteinase K is a stable and highly reactive serine protease which, according to the product information provided by Sigma-Aldrich, retains its activity within a broad pH (pH optimum of 7.5, pH maximum of 12) and temperature range (25 – 60°C). These characteristics make it particularly suitable for the analysis of forensic samples which often contain thermally labile substances that are unstable at pH extremes. The effectiveness of the enzyme in hydrolysing proteins under different conditions was assessed by thin layer chromatography. Given the instability of cocaine at above neutral pH and at elevated temperature, it was important to ascertain the minimum pH and duration of heating at which the enzyme would still be effective. Method development was carried out using lean beef obtained from a butcher. Tissue homogenates (2 g, prepared at a 1:3 tissue to 1.0 M phosphate buffer ratio) were incubated at 37°C at pH 6.5 and 7.5 for 1, 1.25, 1.5, 1.75 and 2 hours following addition of 4 mg proteinase K (1 mg/g of tissue). Thin layer chromatography results indicated comparable levels of protein hydrolysis for all conditions studied, as determined from number of bands corresponding to individual amino acids and the relative intensity of band colour. In selecting pH condition, the stability of cocaine in buffer was assessed by heating replicate samples at 37°C for one hour. Cocaine was significantly more stable at

pH 6.5 (3% cocaine loss) compared with pH 7.5 (18% cocaine loss). Subsequent experiments involved buffering the homogenate at pH 6.5 and heating for one hour.

Drug recovery following enzyme digestion was compared to that obtained following mechanical homogenisation alone. All digested and non digested homogenates were diluted with 8 mL 0.1 M phosphate buffer (pH 6.0) and centrifuged. The supernatants were used for SPE. A major problem with the enzyme digested samples was immediately evident. The supernatant could not be poured off due to a sludgy residue at bottom of centrifuge tube (solid pellet did not form). The supernatant instead had to be pipetted off the top avoiding contact with the sludgy pellet. It was visible that a significant portion of the supernatant was left behind and what portion of the sample was retrieved was very cloudy. The sample had to be pulled through the SPE cartridge with full vacuum and in some samples the cartridge became clogged and the sample had to be discarded. In the first experiment the relative recovery of cocaine and 6AM (as compared with direct extraction) was 37 and 50% respectively. Initially a higher concentration of enzyme was added to samples in the hope a more complete digestion would occur resulting in a clearer digest. No difference in the consistency of the digest was observed. Subsequent experiments involved filtering the digest through filter paper both pre- and post-centrifugation. This took a considerable amount of time and in some samples filtering was not complete even after three hours. Samples were then filtered through glass wool and although this was considerably more rapid, the resulting filtrate was still cloudy. Full vacuum was required for an extended period although on occasion samples could not be pulled through and had to be discarded. The clogging of these cartridges could be partly attributed to the glass wool, which was clearly visible on the surface of the sorbent bed. Although recovery was slightly better following glass wool filtering, it was still only around 60% that obtained by direct extraction. Samples that underwent direct extraction produced clearer supernatants. The clearest supernatants were obtained when the homogenate was sonicated (15 minutes) prior to buffer dilution and centrifugation. These samples flowed through the columns under gravity. Owing to time constraints on the further development of the enzyme digestion method the direct extraction method (with sonication) was chosen for use in the current assay.

3.7.4 Hydrolysis of conjugates

The ratio of parent drug to metabolite is an important factor in the interpretation of toxicology results. Morphine and codeine both form conjugates with D-glucuronic acid to form their respective β -D-glucuronide phase II metabolites. Free morphine to glucuronide concentration ratios in a fresh cadaver are of particular importance since they can give an indication of survival time following drug administration (Bogusz et al. 1997).

These phase II conjugated metabolites are, by definition, very polar and thus not amenable to direct GC-MS determination. In order to measure conjugates indirectly, that is in conjunction with independent measurement of the free drug, either selective (enzyme) or non-selective (acid) hydrolysis of the sample is undertaken to achieve conjugate cleavage. Incubation with strong mineral acid, i.e. hydrochloric acid, is a rapid, inexpensive method which gives non-selective hydrolysis of conjugates. Following hydrolysis, samples must be neutralised to prevent the rapid deterioration of the chromatography column. Moreover, correct pH adjustment is vital to the drug extraction stage. In contrast, incubation with β -glucuronidase (EC 3.2.1.31) gives selective hydrolysis of β -D-glucuronides under relatively mild conditions. Generally cleaner extracts result but the method incurs additional cost and time for incubation. The pH and temperature optima of the specific enzyme preparation are important considerations to ensure maximum activity and hydrolytic efficiency.

Owing to the reduced cost associated with acid hydrolysis, this method was initially tested for use in the current total morphine (TM) assay. However, great difficulty was experienced in neutralising the pH and the resulting extracts produced excessively noisy chromatograms and poor chromatographic behaviour of the morphine peak. As a result development of an acid hydrolysis method was not pursued and enzyme hydrolysis was chosen for the current assay. β -glucuronidase from *Helix pomatia* (*H. pomatia*), *Escherichia coli* (*E. coli*), *Patella vulgata*, *Helix aspersa*, abalone entrails and bovine liver have been shown to hydrolyse morphine conjugates (Fish and Hayes, 1974; Bowie and Kirkpatrick, 1989; Huang et al. 1992; Jennison et al. 1993; Zezulak et al. 1993; Lin et al 1994; Romberg and Lee, 1995; Crandall et al. 2006b; Duflou et al. 2009). Probably the most commonly employed source is from *E. coli*, although in comparison to the

other enzyme sources this is an expensive option. Owing to the lower cost of *H. pomatia*, this source of β -glucuronidase was chosen for use in the current assay.

As a starting point for method development, 10,000 units (U) of β -glucuronidase (Type H-1 from *H. pomatia*; Sigma-Aldrich, Pool, UK) was added to blood (1 mL) following pH adjustment to pH 5.0 (the optimum enzyme pH as reported by the manufacturer) with 1 M acetate buffer (1 mL) and incubated at 37°C overnight (16 hours). These temperature and incubation conditions have been reported previously for *H. pomatia* in the analysis of TM in human umbilical cord (Rawal et al. 2007). To determine the hydrolysis efficiency, horse blood samples were spiked with morphine-3-glucuronide (M3G) at low (n = 6) and high (n = 6) concentrations (0.17 and 0.67 mg/L), which at 100% hydrolytic efficiency would produce free morphine at concentrations of 0.1 and 0.4 mg/L respectively. This experiment was repeated at half the concentration of enzyme (5,000 U per mL of blood). The results showed that both concentrations of enzyme hydrolysed between 88 and 91% of M3G at both the high and low spike concentrations (Table 9). In later experiments on muscle tissue, similar hydrolytic efficiencies were obtained although the efficiency was slightly better at lower enzyme concentration (Table 9). The lower enzyme concentration was chosen for use in the final assay.

Since this method was validated for use in cases involving heroin, the TM measured could have an additional source if the heroin metabolite, 6AM, undergoes degradation during incubation. The stability of 6AM was tested under the assay conditions by spiking blood samples with 150 ng 6AM (n = 8) and measuring the concentration at time 0 (n = 4) and again following heating for 16 hours at 37°C (n = 4). There was no notable difference between the concentration of 6AM measured at time 0 (0.16 mg/L) and at the end of the 16 hours heating period (0.15 mg/L). Morphine was not produced in any sample by the end of the heating period.

Shortly following the preliminary method development at 37°C a publication was discovered which reported the optimum temperature condition for β -glucuronidase from *H. pomatia* to be 50 - 60°C (Romberg and Lee 1995). By increasing the incubation temperature the incubation time could have been reduced. However, prior to the continuation of the method development, technical problems resulting in significant

instrument down time, as well as time restrictions upon the analysis of real case samples, meant that the temperature aspect of the method did not undergo further development. Moreover, heating at a higher temperature might have resulted in breakdown of 6AM to morphine. The final assay was fully validated prior to use and more than acceptable in terms of hydrolytic efficiency and assay precision (Section 3.9). Validation of the TM assay was carried out according the plan outlined in Table 10.

Table 9 Morphine-3-glucuronide (M3G) hydrolysis efficiency in spiked blood and tissue homogenates with β -glucuronidase added at two concentrations

Matrix	β -gluc (units/mL sample)	M3G spike (mg/L)	Maximum FM concentration (mg/L)	Measured FM concentration (mg/L)*	Precision (%)	Efficiency (%)
Blood	5,000	0.17	0.10	0.091	11	91
	5,000	0.67	0.40	0.36	6	91
	10,000	0.17	0.10	0.088	6	88
	10,000	0.67	0.40	0.37	7	92
Muscle	5,000	0.17	0.10	0.093	11	93
	5,000	0.67	0.40	0.40	6	99
	10,000	0.17	0.10	0.085	8	85
	10,000	0.67	0.40	0.34	7	85

* Mean of six replicate analyses

FM: free morphine; M3G: morphine-3-glucuronide; β -gluc: β -glucuronidase

3.8 Validity of case results

In addition to the full validation of an assay, quality control procedures must be routinely carried out during batch analyses to assure the reliability of the analytical data produced. In the current method two quality control (QC) standards were employed in order to determine the validity of the calibration across the concentration range. A low (LQC) and high (HQC) concentration standard was prepared from purchased reference material and weighed or measured separately from the calibrators. A blank matrix sample was used as a negative control to ensure batch contamination had not occurred. As with the calibrators the QC's were matrix matched, apart from for vitreous where aqueous QC's were employed. A maximum deviation of $\pm 20\%$ of the mean of the nominal QC value was deemed acceptable to ensure the validity of the calibration

(Cooper et al. 2010). A single set of QC's and a blank were run concurrently with each SPE batch to confirm the validity of real case samples.

In instances where the concentration of an analyte exceeded that of the highest calibrator, or was less than that of the lowest calibrator, an appropriate volume was re-extracted, sample volume permitting. In either instance, where additional sample was unavailable, the specimen was reported as containing the analyte at a concentration greater or lower than that of the highest or lowest calibrator respectively. For specimens having concentrations significantly higher than the highest calibrator, checks were made to ensure carry-over of analyte into the next specimen had not occurred. Similarly, specimens with very low concentrations were checked to ensure that carry-over from a previous very high positive had not occurred.

Outlier results of case specimens may not be identified if only run singly. For this reason replicate extraction and quantitative analysis was carried out in duplicate when sufficient sample volume was available. Ideally, extractions would have been carried out in triplicate but owing to the cost of SPE cartridges this was not financially viable. Where the possibility of carryover from a higher concentration sample into one of the extracts was excluded, a maximum deviation of $\pm 20\%$ of the mean concentration of the two extracts was deemed acceptable to ensure the validity of the result.

3.8.1 Quantification in vitreous humour

Although matrix matched calibrators are recommended when dealing with biological specimens (Cooper et al. 2010), the use of animal vitreous is impractical for routine use since removing the vitreous from the eye is time consuming and often only a very small volume can be extracted. Drug quantification in vitreous specimens is typically carried out using calibration curves generated in water (e.g. Ziminski et al. 1984; Stephen et al. 2006), plasma (e.g. Wyman and Bultman 2004) or blood (e.g. Duer et al. 2006; Antonides et al. 2007). Given that vitreous is composed of 99% water (Forrester et al. 1996), this matrix was considered a suitable representative for the validation of the vitreous assay. To determine the accuracy of vitreous quantification using aqueous standards, sheep vitreous was spiked with each analyte at low and high concentration ($n = 3$) and quantified using a concurrently run aqueous calibration curve. Results were all within 15% of the nominal value confirming water to be a suitable matrix for the quantification of VH.

3.9 Method Validation

3.9.1 Summary

A GC-ion trap-MS/MS method was developed and validated for the simultaneous extraction and quantification of cocaine, BZE, COET, morphine, TM, codeine and 6AM in blood (from horse), muscle tissue (from deer) and in water as an analogue for VH. Samples were prepared by the addition of deuterated internal standards and buffer dilution. Analytes were extracted from all matrices using solid phase extraction (SPE) with mixed mode sorbent (HCX). Extracts were evaporated and derivatised with BSTFA + 1%TMCS. Total morphine analysis was carried out in blood and muscle following hydrolysis with β -glucuronidase. Chromatographic data were obtained on a BPX5 fused silica capillary column. Spectral data were obtained by electron impact ionisation in the tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM) modes. Compound identification was achieved by comparison of retention time and full product ion spectral data to that obtained from concurrently run reference standards. The assay was validated for linearity, limit of quantification, repeatability, intermediate precision, accuracy and analytical recovery in each matrix. The limit of quantification (LOQ) was ≤ 0.01 mg/kg in muscle and ≤ 0.005 mg/L in blood and water. Good linearity was observed over the concentration ranges studied ($R^2 \geq 0.99$). The repeatability (%RSD) at the three concentration levels was typically $\leq 15\%$ and never exceeded 17%. Intermediate precision of $\leq 16\%$ was obtained for all matrices. Deviation from the nominal concentration was always less than 20% in repeatability and intermediate precision experiments. Extraction recoveries for opiates ranged from 59-104% in water, 50 – 95% in blood and 41 – 79% in muscle. Recoveries for cocaine and metabolites ranged from 91 – 110% in water, 81 – 110% in blood and 61 – 75% in muscle. The hydrolysis efficiency for the TM assay in blood and muscle tissue ranged from 91 – 99% with within-day and intermediate precisions of $\leq 14\%$ and $\leq 12\%$ respectively.

3.9.2 Methods

3.9.2.1 Chemicals and reagents

Cocaine, BZE, COET, morphine, codeine, 6AM and M3G were purchased from either Sigma-Aldrich (Poole, UK) or LGC Standards (Teddington, UK). Internal standard

solutions of cocaine-d3, BZE-d3, morphine-d3 and 6AM-d3 (100 µg/mL) were purchased from LCG Standards. All drug standards were stored according to the manufacturer's instructions. BSTFA + 1% TMCS and β-glucuronidase, Type H-1 (from *Helix pomatia*), were purchased from Sigma-Aldrich. Concentrated hydrochloric acid (37%), ammonia solution (33%) and all other reagents and solvents were of analytical grade and obtained from Fisher Scientific (Loughborough, UK). 1 M phosphate buffer (pH 6.0) was prepared with the addition of 2% sodium fluoride (NaF) (Fisher Scientific, Loughborough, UK). ISOLUTE® Confirm SPE cartridges (130 mg HCL sorbent, 10 mL volume; Cat# 902-0013-H) were generously provided by Biotage (Uppsala, Sweden).

3.9.2.2 Specimens

Oxalated horse blood (TCS Biosciences, Buckingham, UK) and skeletal muscle obtained from the hind quarter of freshly culled Sika Deer (New Forest, Dorset, UK) were used for validation of the assay. Autopsy materials (blood and muscle) that had screened negative for drugs of abuse were also used in the determination of method selectivity.

3.9.2.3 Standard solutions

Methanolic stock solutions containing all of the non-deuterated analytes, with the exception of 6AM, were prepared at concentrations of 100, 10 and 1 mg/L from individual 1 mg/mL solutions. 6AM was prepared separately at the same concentrations. Calibration standards were prepared in deionised water from the 100, 10 and 1 mg/L methanolic solutions. QC and validation working solutions were prepared at the required concentration (Table 10) in water from independently prepared 100, 10 and 1 mg/L methanolic stock solutions. For recovery determination validation standard solutions were prepared at the same nominal concentration in methanol. The internal standard working solution containing all deuterated analytes was prepared in water from 100 mg/L stock solutions (as purchased) at a concentration of 1.5 mg/L cocaine-d3, BZE-d3 and morphine-d3 and 0.4 mg/L 6AM-d3. Stock solutions of M3G were prepared in water at concentrations of 1.7 and 6.7 mg/L. Working solutions were prepared to give a concentration at which the final spike volume was 100µL.

3.9.2.4 Sample preparation

For validation, calibration and QC purposes 100 μ L of the appropriate standard solution was added to 1 mL blank oxalated horse blood / 1mL water containing 2% NaF. Blood samples were rotated on a rotary mixer for 30 minutes to ensure even distribution of the standard throughout the sample. To prepare the muscle homogenates, tissue was first macerated using scissors and any visible fat or connective tissue was removed. The tissue was weighed and 1.0 M phosphate buffer (pH 6.0) / 2% NaF was added at a weight three times that of the tissue weight. The tissue was homogenised using a PowerGen 125 homogeniser (Fisher Scientific, Loughborough, UK) at 18,000 rpm for three minutes and then at 22,000 rpm until the sample was smooth and homogeneous. Two grams of homogenate (equivalent to 0.5g tissue) was weighed into 15 mL polypropylene centrifuge tubes and spiked with 100 μ L of the appropriate standard. Owing to the greater viscosity of muscle homogenates these samples were not rotated but instead vortex mixed at high speed for 20 seconds.

With the exception of blank samples, 100 μ L of internal standard (IS) working solution, prepared to give a final concentration of 0.15 mg/L cocaine-d3, BZE-d3 and morphine-d3, and 0.04 mg/L 6AM-d3, was added to all matrix matched validation samples, calibration and check standards. All samples were vortex mixed. To assist the SPE extraction blood and tissue homogenate samples were also sonicated for 15 minutes. Following the addition of 8 mL 0.1 M phosphate buffer (pH 6.0) all samples were again vortex mixed. Blood and muscle samples were then centrifuged at 2,500 and 3,000 rpm respectively, for 12 minutes. Blood and tissue supernatants and the whole water sample were used for SPE.

3.9.2.5 Analysis of total morphine (TM)

For TM analysis, 1 mL blood / 2 g muscle tissue homogenate was spiked with M3G to achieve the required concentration (Table 10). Blood samples were then rotated and tissue homogenates vortex mixed as described earlier. Morphine-d3 internal standard solution was added to each sample to achieve a final IS concentration of 0.15 mg/L. Samples were sonicated for 15 minutes. 1 mL of 1.0 M acetate buffer (pH 5.0) and 100 μ L of β -glucuronidase solution (50,000 U/mL in distilled water) were added to each sample (5,000 U of enzyme per sample). Tubes were capped, vortex mixed and incubated at 37°C for 16 hours. Samples were allowed to cool to room temperature and

then treated with 8.0 mL 0.1 M phosphate buffer (pH 6.0) and centrifuged as described earlier for blood and tissue. The supernatant was used for SPE.

3.9.2.6 Solid phase extraction

SPE cartridges were conditioned by sequential addition of 2 mL methanol and 2 mL 0.1 M phosphate buffer (pH 6.0). Samples were introduced on to the columns and allowed to flow under gravity. Columns were washed by sequential addition of 6mL deionised water and 3 mL 0.1 M HCl. Columns were dried under full vacuum for 5 minutes followed by an additional wash of methanol (6 mL). Columns were dried for a further 5 minutes under full vacuum. Samples were eluted with 2 mL of fresh dichloromethane/isopropanol/ammonium hydroxide (80:18:3 v:v:v) at a flow rate of approximately 0.1 mL/minute and collected in conical bottom glass tubes. Extracts were evaporated to dryness under a gentle stream of nitrogen at $\leq 40^{\circ}\text{C}$.

3.9.2.7 Derivatisation

Dried residues were reconstituted in 25 μL ethyl acetate and 25 μL BSTFA +1% TMCS, capped, vortex mixed and heated in an incubator for 20 minutes at 70°C . To complete the derivatisation process samples were allowed to stand at room temperature for 2 hours prior to transfer into autosampler vials.

3.9.2.8 GC-MS/MS analysis

Gas chromatographic analyses were performed on a Varian 2200 gas chromatograph equipped with a Varian Saturn 2000 ion-trap detector (Agilent Technologies, Stockport, UK). A BPX5 fused silica capillary column (30 m \times 0.25 mm i.d., 0.25 μm film thickness) with 5% phenyl – 95% methyl-polysiloxane stationary phase was obtained from SGE Analytical Science (Ringwood, Australia). Ultra high purity helium was used as carrier gas at a flow rate of 1.0 mL/minute. The column oven temperature was programmed from an initial temperature of 130°C held for one minute, ramped to 240°C at a rate of $30^{\circ}\text{C}/\text{minute}$ held for four minutes, ramped to 260°C at $15^{\circ}\text{C}/\text{minute}$, held for two minutes, then finally to 300°C at $60^{\circ}\text{C}/\text{minute}$ held for 3.33 minutes (total run time of 16 minutes). 2 μL of sample was injected via on column injection using the Varian 8200 Autosampler (Agilent Technologies, Stockport, UK). The injection port temperature was maintained at 260°C . Transfer line, manifold and trap temperatures were set at 260, 100 and 200°C respectively. The filament emission current and

multiplier offset were set at 50 μ A and 300 mV respectively. A mass defect of 100mmu/100u was set for each compound. The ion-trap was operated in MS/MS mode for codeine and in multiple reaction monitoring (MRM) mode for all other compounds. Ions were isolated using the non-resonant waveform with an isolation window and excitation storage level set at 3.0 m/z and 75 m/z respectively. Collision induced dissociation (CID) and compound specific parameters are reported in Table 5. Quantification ions and internal standard allocations are also reported. All aspects of data acquisition and data processing were controlled using the Varian Saturn 2000 MS software. Analyte identity was confirmed if the match threshold value was $\geq 90\%$ and the retention time data was within ± 0.1 minutes when compared to concurrently run reference standards.

3.9.3 Validation parameters

3.9.3.1 Method selectivity

Ion preparation methods such as MS/MS can ensure specificity for the analyte of interest. However, peaks originating from endogenous compounds and/or contamination may be problematic with MS/MS analysis if a co-eluting substance is present in high enough concentration. It is therefore necessary to examine the regions of chromatographic interest in the blank matrix over a larger mass range. Several sources of blank human and animal blood and muscle tissue were run in full mass scanning mode (80 – 600 m/z) using the MS/MS assay temperature program and examined for peaks that could potentially interfere with the MS/MS assay. Blank samples were then re-run using the MS/MS method and the detection windows were examined for ion interference. Selection of the quantification ions was based on i) no significant ion interference; ii) ion abundance at either 100% (base peak) or $> 40\%$ the abundance of the base peak, and; iii) good peak shape (narrow and symmetrical).

Table 10 Validation Plan

Validation parameter	Acceptance Criteria	Experimental design
Method selectivity	No interfering ions within 0.2 min of the analyte peak retention time	Selectivity is determined by processing three independent sources of blank matrix. The chromatograms are examined across each MS/MS analyte time segment. Selectivity is assessed by the presence of ions in the matrix having the same m/z as those used for quantification (interfering ions).
Linearity (R^2)	≥ 0.99	The coefficient of determination (R^2) is determined in triplicate over the range 0.01 – 1.0 mg/L for cocaine, BZE, COET, codeine and morphine and; 0.005 – 0.2 mg/L for 6AM. Curves run on three different days.
Repeatability (% RSD)	$\leq 15\%$ (medium and high concentration) $\leq 20\%$ (low concentration)	Six replicate analyses performed in the matrix spiked at low (6AM: 0.01 mg/L; other analytes: 0.025 mg/L), medium (0.05; 0.15 mg/L) and high (0.1; 0.4 mg/L) concentration and quantified with a concurrently run calibration curve. The %RSDs of the results at each concentration level is calculated.
Intermediate precision (% RSD)	$\leq 20\%$	Three replicates analysed at each concentration on two different days using different batches of standard solutions and carried out by a different analyst. The %RSD is calculated from the mean and SD data obtained from all three repeatability experiments.
Accuracy (% deviation)	+/- 20%	Using the data obtained from repeatability experiments accuracy is calculated as the deviation of the mean measured concentration to the nominal value (spike amount).
Recovery (%)	NA (pre-defined precision and accuracy criteria are met)	Recovery is determined concurrently with repeatability and accuracy experiments at low, medium and high concentrations. The peak response obtained from extracted standards is compared to that obtained for the same quantity of pure standard spiked into blank extracted matrix (calculated as a percentage).
LOQ (mg/L; mg/kg)	RSD of result $\leq 20\%$ All results +/- 20% nominal	The LOQ is defined as the lowest concentration for which the variation (%RSD) of the result over six replicate analyses is $\leq 20\%$, the measured concentration falls within +/- 20% of the nominal for each replicate, and the qualification criteria are met (Rt within 1% of calibrator; match threshold $> 90\%$). Each replicate (1mL blood/1mL water/2g tissue homogenate (=0.5g tissue)) is spiked with 10, 7.5, 5, 2.5 and 1 ng of cocaine, COET, BZE, codeine and morphine and 5, 4, 3, 2 and 1 ng of 6AM.
M3G hydrolysis efficiency (% hydrolysis)	$\geq 70\%$ (repeatability and intermediate precision criteria are met)	Six replicate TM analyses performed in blood and muscle tissue on two days at low (0.17 mg/L) and high (0.67 mg/L) M3G concentration. Hydrolytic efficiency is the measured free morphine concentration expressed as a percentage of the maximum free morphine concentration (0.1 and 0.4 mg/L). The calculation of and acceptance criteria for repeatability and intermediate precision are as described above.

3.9.3.2 Linearity

The concentration range chosen for the assay was based on the concentration ranges anticipated in real case work and on preliminary linearity experiments using curves generated in standard solution (Section 3.6.1). A seven-point calibration over the concentration range 0.01 – 1 mg/L (mg/0.5 kg) for cocaine, BZE, COET, codeine and morphine and 0.005 – 0.2 mg/L (mg/0.5 kg) for 6AM was chosen for the final assay (Table 10). Quality control samples were prepared using independently prepared standard solutions at 0.03 and 0.4 mg/L (mg/0.5 kg) for cocaine, BZE, COET, codeine and morphine and 0.0125 and 0.04 mg/L (mg/0.5 kg) for 6AM and run concurrently with the curves to determine the validity of the calibration. Curves were processed in triplicate and each run on a different day. Linearity was determined using least squares regression with $1/x^2$ weighting to compensate for heteroscedasticity. A coefficient of determination (R^2) ≥ 0.99 was deemed acceptable.

3.9.3.3 Accuracy and precision

Accuracy and repeatability (within day precision) were assessed simultaneously by spiking six replicate matrix matched blank specimens at low, medium and high concentration (Table 10). Intermediate (between-day) precision was assessed by repeating the precision experiment with three replicates on an additional two days using different standard solutions and carried out by a different analyst. Accuracy was calculated as the percentage deviation of the mean of the measured concentration from the nominal concentration; $(\text{nominal concentration} - \text{mean measured concentration})/\text{nominal concentration} \times 100$. Repeatability was calculated as the percentage relative standard deviation; $(\text{standard deviation of the measured mean}/\text{measured mean}) \times 100$. The acceptance criteria for precision and accuracy are set out in Table 10.

3.9.3.4 Recovery

Recovery was assessed at low, medium and high concentration by directly comparing the response of extracted accuracy/precision standards with that obtained from extracted blank matrix to which analyte was added at the same nominal concentration expressed as a percentage (Table 10). In this way any matrix effects do not complicate interpretation of recovery data (Dadgar et al. 1995).

3.9.3.5 Limit of quantification

The limit of quantification (LOQ) of an assay may be defined as the lowest amount of analyte in a sample which can be quantitatively determined at a pre-defined level of accuracy and precision (Hubert et al. 2004). The LOQ was defined as the lowest concentration at which the repeatability was $\leq 20\%$ (RSD) and the pre-defined accuracy ($\pm 20\%$ deviation from nominal) was met in 100% of the analyses. The LOQ was determined experimentally by analysing replicate samples containing progressively lower amounts of the analyte (Table 10).

3.9.3.6 Total morphine assay: efficiency and precision

For TM analysis matrix matched calibration standards containing only morphine were prepared over the range 0.01 – 1.0 mg/L. QC samples containing M3G at low and high concentration were prepared in blood and muscle tissue (Table 10). Hydrolytic efficiency was defined as the post-hydrolysis morphine concentration expressed as a percentage of the maximum possible morphine concentration. Repeatability and intermediate precision (over two days) was calculated as described earlier.

3.9.4 Results and discussion

3.9.4.1 Method selectivity

On examination of several sources of human blank matrix over the full mass range, no major interference was observed in the specific time detection windows of the analytes of interest. Examination of the detection windows using the MS/MS method showed some low level ion interference ± 1 m/z of the precursor ion mass. This was observed particularly in some blood and muscle samples for cocaine (182 m/z) and BZE (240 m/z). The lower mass 150 m/z product ion showed no interference in either time segments and demonstrated superior peak shape and greater sensitivity. The parent and precursor ion of morphine (429 m/z) is also a commonly observed column bleed ion and so resulted in an elevated background in this detection window. Similarly a raised background was observed in the 6AM detection window for the precursor ion (399 m/z). Specificity for each analyte was ensured by selection of the next most abundant ions for use in quantification (Table 5); 234 m/z for morphine and 340 m/z for 6AM. The 234 m/z ion was also present in high abundance for codeine and was selected as the quantification ion for this compound as it demonstrated superior peak shape compared to the 370 m/z base peak. Ion chromatograms obtained for each analyte in the validation

matrices are displayed in Appendix 2 (water), 3 (horse blood) and 4 (deer tissue). Chromatograms for human case material containing the target analytes are presented for vitreous humour in Appendix 5 (opiates) and 6 (cocaine), for blood in Appendix 7 (opiates) and 8 (cocaine) and in Appendix 9 for muscle tissue (cocaine). Unfortunately opiate positive human muscle was not available in this study.

3.9.4.2 Linearity

In each of the matrices studied all compounds demonstrated good linearity over the range analysed ($R^2 \geq 0.99$). The final calibration ranges, linear regression data and corresponding coefficients of determination (R^2) are presented in Table 11. Quantification of the LQC and HQC and the back calculated concentrations of the calibration standards were all within 20% of the nominal value. Calibration curves for each analyte are displayed in Appendix 10 (aqueous), 11 (blood) and 12 (muscle).

3.9.4.3 Accuracy and precision

Accuracy, repeatability and intermediate precision results for each analyte in water, blood and muscle tissue are displayed in Tables 12, 13 and 14 respectively. The raw data for all analyses, including intermediate precision data, are presented in Appendix 13 (aqueous), 14 (blood) and 15 (muscle). Percentage deviation from the nominal (accuracy) was within the 20% deviation acceptance limits for each matrix with maximum deviations of 11, 16 and 19% obtained in water, blood and muscle respectively. The repeatability was generally $\leq 15\%$ in all matrices and at all concentrations. The only exception was for morphine measured at low concentration in blood and muscle for which the repeatability was 17%. There was no general trend toward lower precision and/or accuracy at low concentration. Intermediate precision was well within the accepted 20% and did not exceed 16% in any matrix.

3.9.4.4 Recovery

Extraction recoveries obtained in water, blood and muscle tissue are displayed in Tables 12, 13 and 14 respectively. Cocaine and metabolite recovery ranged from 91 – 110% in water, 81 – 110% in blood and 61 – 75% in muscle. Opiate recovery ranged from 59 – 104% in water, 50 – 95% in blood and 41 – 79% in muscle. For all analytes the percentage recovery in each matrix generally decreased as analyte concentration increased. In general it is most important to reach a reproducible recovery, which is high

enough to satisfy the requirements of detecting and/or quantifying low sample concentrations, even when the recovery itself is low (Hartmann et al. 1998). Although the recovery of the opiates from each matrix, particularly morphine and 6AM, was lower than for cocaine, BZE and COET, it was acceptable as the precision and accuracy criteria were met.

3.9.4.5 Limit of quantification

The LOQ for each analyte, as determined from the pre-defined criteria, are displayed in Table 15 with the corresponding accuracy and precision data. Concentration data is displayed as ng/g for muscle (multiplied up from ng/0.5g) and ng/mL for water and blood. The LOQ for each analyte was determined to be at a concentration lower than that of the lowest calibration standard used in the assay. Raw data for all analyses are presented in Appendix 16 (aqueous), 17 (blood) and 18 (muscle).

3.9.4.6 Total morphine assay: efficiency and precision

The hydrolysis of M3G in blood and muscle tissue ranged from 91 – 99%. The repeatability and intermediate precision in blood and muscle tissue was $\leq 14\%$ and $\leq 12\%$ respectively (Table 16). Raw data for all analyses including intermediate precision data are presented in Appendix 19 (blood) and 20 (muscle).

3.9.4.7 Putrefied or atypical samples

Although the samples analysed in the present study were all relatively fresh, it is acknowledged that the application of this method to putrefied and atypical samples may be problematic with respect to the reliable measurement of the target analytes. Decomposed blood and tissue create a significant challenge because the high concentration of lipids and putrefactive amines may obscure or interfere with detection and/or accurate quantification of target analytes. As a result of the chemical similarity of putrefactive amines to the basic analytes of interest investigated here, these interfering compounds would be similarly retained on the HCL column and eluted at the high pH of the elution solvent. Whilst the high selectivity of the MS/MS detection method may be effective in the elimination of matrix interferences, there may be a sufficient matrix effect to adversely influence quantitative measurement. Overcoming the effects of specimen decomposition is challenging because samples vary greatly in their extent of decomposition and finding a blank matrix-matched sample for the preparation of

calibration standards or controls is therefore extremely difficult. The quantitative determination of analytes in decomposed or other deteriorated samples is invariably less accurate than in fresher samples (Moffat et al. 2004) and this must be considered when interpreting analytical results obtained from such material. In general fewer problems should be encountered with VH since the interior of the eye is reported to be a sterile medium until the most advanced stages of decomposition (Moffat et al. 2004) and thus this fluid does not undergo putrefaction as would blood and other tissues.

Decomposition may also interfere with the general extraction procedure as a result of the condition of the tissue and subsequent sampling difficulties, e.g. blood sediments and unevenly clots post-mortem with a decrease in blood water content (Skopp 2004). This not only affects the ease of sampling but also the resulting measured concentration. Muscle tissue that has a high fat content (often observed in the elderly) is extremely difficult to analyse by SPE. In the present study muscle preparation difficulties were encountered in two cases where opiate ingestion prior to death was suspected in elderly decedents (blood screening later revealed both cases to be negative for relevant opiates). The supernatants obtained from the muscle samples in each case were extremely cloudy, presumably the result of lipid-in-aqueous emulsion formation. Some of these samples clogged the SPE cartridges precluding subsequent analysis. A modification of the present muscle preparation procedure and subsequent re-validation would be necessary to improve the analysis in these types of cases, e.g. prior to SPE a lipid removal step could be employed by extracting with organic solvent. At the pH of the buffer utilised for homogenisation the target analytes would be ionised and remain in the aqueous phase and the lipids discarded with the organic phase. Often, modifications of routine analytical methods, and in some instances the development of completely new extraction and detection methods, are necessary to enable the reliable measurement of analytes in decomposed, deteriorated or atypical biological samples frequently encountered in post-mortem cases (Skopp et al. 2004).

3.9.5 Conclusions

This validated procedure offers a simple, sensitive and highly selective assay for the analysis of key opiates and cocaine in blood, vitreous humour and muscle tissue. The use of ion-trap operated in MS/MS mode provides a simple means of removing matrix interferences without the loss of mass spectral information associated with single ion monitoring. In contrast to the use of ion ratios, where much of the mass spectral data is effectively discarded, analyte confirmation can be achieved by comparison of the full product ion spectrum to that obtained from reference standards.

Table 11 Linear range and regression data

Matrix		Cocaine	COET	BZE	Codeine	Morphine	6AM
Linear range (mg/L / mg/0.5kg)		0.01 – 1.0	0.01 – 1.0	0.01 – 1.0	0.01 – 1.0	0.01 – 1.0	0.005- 0.2
Water	Coefficient of determination	0.9998	0.9968	0.9999	0.9965	0.9956	0.9928
	Slope	1.2728	0.8472	0.8975	1.3499	0.8867	1.1123
	Intercept	0.0457	0.0083	0.0381	0.0343	0.0040	0.0079
Blood	Coefficient of determination	0.9995	0.9958	0.9999	0.9976	0.9983	0.9982
	Slope	1.0770	0.8788	1.3000	2.1143	0.6578	1.0221
	Intercept	0.0726	0.0155	0.0199	0.0202	0.0205	0.0194
Muscle	Coefficient of determination	0.9967	0.9988	0.9983	0.9967	0.9985	0.9992
	Slope	0.9411	0.7150	0.9637	2.8332	0.9012	0.9944
	Intercept	0.022	0.0029	0.0128	0.0426	0.0047	0.0381

Table 12 Accuracy, precision and recovery in water

Compound	Nominal Concentration (mg/L)	Measured concentration (mg/L)*	Accuracy (%)	Repeatability (%)	Intermediate precision (%)	Recovery (%)
Cocaine	0.025	0.026	5	5	6	107
	0.15	0.15	1	5	7	91
	0.40	0.44	11	4	14	99
COET	0.025	0.028	11	9	11	110
	0.15	0.15	1	10	11	91
	0.40	0.43	7	8	8	92
BZE	0.025	0.025	0	7	8	107
	0.15	0.14	9	10	17	107
	0.40	0.37	7	6	10	91
Codeine	0.025	0.023	8	5	8	104
	0.15	0.14	10	5	13	102
	0.40	0.36	10	5	10	88
Morphine	0.025	0.025	1	4	9	93
	0.15	0.15	0	6	10	85
	0.40	0.36	9	5	6	72
6AM	0.010	0.010	0	9	9	79
	0.050	0.048	4	10	11	64
	0.10	0.096	4	8	6	59

*Mean of six replicate analyses

Table 13 Accuracy, precision and recovery in blood

Compound	Nominal Concentration (mg/L)	Measured concentration (mg/L)*	Accuracy (%)	Repeatability (%)	Intermediate precision (%)	Recovery (%)
Cocaine	0.025	0.025	1	6	6	104
	0.15	0.15	0.2	7	7	98
	0.40	0.42	4	7	6	94
COET	0.025	0.025	2	8	8	110
	0.15	0.14	5	6	10	98
	0.40	0.42	5	6	6	96
BZE	0.025	0.024	4	9	9	101
	0.15	0.14	10	5	13	97
	0.40	0.40	1	5	8	81
Codeine	0.025	0.023	10	13	13	75
	0.15	0.14	5	10	12	79
	0.40	0.38	6	10	11	92
Morphine	0.025	0.024	5	17	13	95
	0.15	0.17	15	7	8	66
	0.40	0.45	11	10	10	60
6AM	0.010	0.009	13	9	14	71
	0.050	0.058	16	8	8	52
	0.10	0.12	15	13	11	50

*Mean of six replicate analyses

Table 14 Accuracy, precision and recovery in muscle tissue

Compound	Nominal Concentration (mg/0.5kg)	Measured concentration (mg/0.5kg)*	Accuracy (%)	Repeatability (%)	Intermediate precision (%)	Recovery (%)
Cocaine	0.025	0.021	15	8	13	74
	0.15	0.16	3	6	8	65
	0.40	0.43	8	8	10	73
COET	0.025	0.025	1	7	6	71
	0.15	0.16	3	3	10	63
	0.40	0.44	10	8	11	71
BZE	0.025	0.029	16	4	11	72
	0.15	0.14	7	5	10	61
	0.40	0.42	5	4	10	75
Codeine	0.025	0.025	1	12	14	77
	0.15	0.12	19	6	14	74
	0.40	0.34	16	6	9	79
Morphine	0.025	0.029	15	17	12	44
	0.15	0.16	6	11	11	42
	0.40	0.41	3	9	15	41
6AM	0.010	0.011	10	13	11	43
	0.050	0.059	18	8	13	42
	0.10	0.11	13	7	11	44

*Mean of six replicate analyses

Table 15 Accuracy and precision at the LOQ in water, blood and muscle tissue

Matrix		Cocaine	COET	BZE	Codeine	Morphine	6AM
Water	LOQ (ng/mL)	2.5	2.5	2.5	2.5	5.0	2.0
	Measured concentration (ng/mL)*	2.8	3.0	2.7	2.6	5.9	1.6
	Accuracy (%)	10	20	10	5	18	19
	Precision (%)	3	4	8	7	1	10
Blood	LOQ (ng/mL)	5.0	1.0	5.0	5.0	5.0	3.0
	Measured concentration (ng/mL)*	4.9	0.95	5.1	5.6	5.7	2.8
	Accuracy (%)	1	8	1	11	15	7
	Precision (%)	11	14	12	7	3	13
Muscle	LOQ (ng/g)	5.0	10	10	10	10	6.0
	Measured concentration (ng/g)*	5.8	11	11	11	11	6.0
	Accuracy (%)	15	6	10	15	8	0
	Precision (%)	16	9	13	10	7	16

*Mean of six replicate analyses

Table 16 Efficiency and precision of the total morphine assay

Matrix	M3G concentration	Maximum free morphine concentration	Measured concentration*	Efficiency (%)	Precision (%)	Intermediate precision (%)
Blood (mg/L)	0.17	0.10	0.091	91	11.4	10
	0.67	0.40	0.36	91	5.9	9
Muscle (mg/0.5kg)	0.17	0.10	0.093	93	11	12
	0.67	0.40	0.40	99	6	11

*Mean of six replicate analyses

Chapter 4.0 The stability of cocaine in horse blood, sheep vitreous and homogenised deer muscle with and without the addition of sodium fluoride

4.1 Abstract

This study examined the *in vitro* stability of cocaine in horse blood, sheep vitreous humour (VH) and homogenised deer muscle stored with, and without, sodium fluoride (NaF). The stability of cocaine in these matrices has not been previously reported. Blood and VH were stored with and without the addition of NaF at 20, 4 and -18°C for 84 days. The effect of NaF concentration (1% and 2%) was determined for blood. Muscle tissue homogenates were prepared in water with and without NaF and also in phosphate buffer (pH 6.0) containing NaF and stored for 31 days at 25, 4 and -18°C. Drug stability in muscle homogenates prepared from authentic human case material was also assessed following storage for 13 months at -18°C. Cocaine and benzoylecgonine (BZE) were extracted using SPE and quantified by GC-ion trap-MS/MS. The stability of cocaine in horse blood appeared to be less than that previously reported in the scientific literature for human blood, an observation that is attributed to the additional hydrolytic pathway provided by carboxylesterase in horse plasma. In the absence of NaF, cocaine could not be detected by day 7 in blood stored at 20°C and 4°C. When blood containing cocaine was stored at -18°C the concentration had declined by 81% after 7 days. In samples containing NaF, cocaine was stable (0% loss) for 7 days at 4°C and for the entire study period at -18°C. At 4°C the rate of cocaine degradation in blood preserved with 2% NaF was found to be significantly slower than with 1% NaF. Sodium fluoride did not have any significant effect on cocaine stability in VH. Significant chemical hydrolysis of cocaine in VH stored at 4°C ($\geq 56\%$) occurred by day 14 and at 20°C losses $\geq 87\%$ were observed by day 7. At -18°C cocaine was substantially more stable. In contrast to blood and VH, cocaine was stable in muscle tissue stored at 4°C and below with a maximum loss of only 2% observed in any sample. In homogenates prepared with 2% NaF and stored at 25°C the concentration of cocaine had declined by only 16% by day 31. Cocaine degraded more substantially in unpreserved and buffered homogenates but could still be detected at the end of the study period. In authentic tissue homogenates cocaine had declined by only 15% following storage at -18°C for 13 months. Muscle provides an excellent matrix for cocaine determination owing to increased stability in this matrix.

4.2 Introduction

Knowledge of drug stability in biological matrices is vital for medico-legal purposes. Drug testing laboratories are required to retain toxicological case material for extended periods in the event that independent analysis or re-analysis of a specimen is requested. The ability to obtain consistent analytical test results following short- or long-term sample storage is essential in establishing the credibility of toxicological data.

The poor stability of cocaine in human blood samples stored without the addition of preservative (e.g. sodium fluoride) and without refrigeration is well documented (Isenschmid et al. 1989; Baselt et al. 1993; Garrett et al. 1994; Skopp et al. 2001a). The stability of cocaine in horse blood has not previously been reported, yet this matrix is available commercially for use in toxicology laboratories and is used extensively in the UK for the preparation of blood calibration and check standards. Knowledge of cocaine stability in this matrix is of particular importance if standards are prepared in bulk and stored prior to use (as is typically the case with quality controls samples which may be stored for up to 6 months). Horse blood, in addition to containing BuChE (Aldridge 1953), contains carboxylesterase (Li et al. 2005), which is known to hydrolyse ester containing drugs such as cocaine (Dean et al. 1991). Thus, the degradation of cocaine in stored horse blood would have an additional enzymatic pathway compared to human blood that might contribute substantially to the breakdown of cocaine in this matrix, particularly if samples are stored without preservative.

It has been reported that in real cases cocaine may be detected for a longer period in the VH than in blood (Mackey-Bojack et al. 2000; Antonides et al. 2007), yet there is currently no data on the short or long-term *in vitro* stability of cocaine in this matrix in either animal or human VH. In a single *in vitro* study conducted in rabbit tissue it was reported that cocaine could be detected for longer in muscle than in blood over a five day period (Moriya and Hashimoto 1996). The stability of cocaine in human or animal muscle tissue stored under different conditions has not been reported.

This study examined the effect of storage temperature and sodium fluoride addition on cocaine stability in horse blood, sheep VH and deer muscle tissue homogenates. Sheep VH and deer muscle were utilised owing to the difficulty associated with acquiring black human samples. The study period spanned 84 days for blood and VH and 31 days

for muscle tissue. Cocaine stability in authentic human muscle tissue homogenates containing sodium fluoride was also examined following storage at -18°C for 13 months. Stability was assessed over time by measurement of the parent compound and its major metabolite, BZE.

4.3 Methods

4.3.1 Specimens

Oxalated horse blood was obtained from TCS Biosciences (Buckingham, UK). Vitreous humour was obtained from sheep within 1 hour of slaughter (Dunbia Abattoir, Carmarthenshire, UK). Samples were aspirated by needle puncture through the sclera. Skeletal muscle was obtained from the hind quarter of freshly culled Sikah deer, (*Cervus Nippon*; New Forest, Dorset, UK). All specimens were stored at -18°C prior to experimental setup.

4.3.1.1 Specimen selection

Owing to restrictions imposed by the Human Tissue Act (1974), the use of human vitreous and human muscle tissue was not practical for this study. It is known that the vitreous gel of the various mammalian species is composed of essentially the same extracellular matrix components (Noulas et al. 2004). Thus, as with human VH, sheep VH predominantly consists of water and does not contain hydrolytic esterases. The sheep VH used in this study was also comparable in terms of pH; the pH of sheep VH following collection was pH 7.6 and in humans the pH of VH is reported to be pH 7.5 (Levine and Jufer 2008). Pig eyes were initially collected for use in this study but yielded a very low volume of VH (~ 1 – 2mL). Sheep VH was chosen for use in this study because the available volume of VH was much higher (approximately 3 – 4 mL per eye). Further, sheep eyes were readily available and free of charge. Skeletal muscle from Sikah deer was the best available specimen for this study because a very fresh sample could be obtained – it was possible to collect the tissue from the animal within 24 hours of death. Further, the animal was free range and not intended for human consumption. This meant the animal had not been subjected to pre- or post-slaughter meat preparation processes that could potentially affect the natural decomposition of the muscle. Further, the pH of deer muscle measured 24 hours following death (pH 5.7

when homogenised with water) was comparable to that previously reported for early post-mortem human skeletal muscle tissue (pH 5.7 – 6.0) (Langford et al. 1998).

4.3.2 Experimental

Horse blood and sheep VH samples were spiked to achieve a final concentration of 0.4 mg/L cocaine. Blood samples were rotated on a rotary mixer for 30 minutes to ensure even distribution of the drug throughout the sample. VH was vortex mixed thoroughly. Blood sample aliquots were prepared in triplicate at each of the three preservative conditions (0, 1 and 2% NaF) and at each of the three study temperatures (20, 4 and -18°C). Sufficient sample volume was stored in each aliquot (4 mL) to enable triplicate extraction at each analysis time (Table 17). Owing to restrictions on sample volume, sheep VH aliquots (1.5 mL) were prepared in duplicate at two preservative conditions (0 and 1.5% NaF) and stored at the same temperatures as for blood. Each VH sample was analysed in duplicate at each analysis time (Table 17). Deer muscle homogenates were prepared with 1) water, 2) water containing 2% NaF, and 3) 1.0 M phosphate buffer (pH 6.0) containing 2% NaF. Following removal of all fat and connective tissue, muscle was segmented with scissors and subsequently homogenised at a 1:3 tissue to buffer ratio using a PowerGen 125 homogeniser (Fisher Scientific, Loughborough, UK). Multiple extractions of a single replicate were not carried out for muscle samples because in preliminary experiments the muscle tissue homogenate became more viscous and inhomogeneous as time of storage increased. Obtaining a homogenous sample was extremely difficult and differences in drug concentration were observed between replicate extractions from the same aliquot. To ensure that concentration differences observed between different storage conditions were the result of differences in stability and not due to inconsistent sampling, muscle stability samples were prepared individually rather than in aliquots. Homogenate samples (2 g; equivalent to 0.5 g tissue) were weighed into a series of polypropylene centrifuge tubes and each spiked individually with 400 ng cocaine. Samples were vortex mixed thoroughly and set up in quadruplet at each preservative condition and stored at 25, 4 and -18°C. Each 2 g homogenate was extracted whole at each of the analysis times (0, 7, 14 and 28 days). Storage conditions and analysis times are presented in Table 17.

The long-term stability of cocaine in human muscle tissue homogenates was also assessed in authentic case samples (n = 14) that were analysed as part of the tissue

distribution study (results presented in Chapter 5) following homogenisation with 1.0 M phosphate buffer containing 2% NaF (preservative condition 3). Each homogenate was extracted in triplicate following storage at -18°C for 13 months.

Table 17 Storage conditions for spiked horse blood, sheep vitreous and deer muscle homogenate

Matrix	Preservative Condition	Replicates (n)	Extractions / replicate (n)	Storage Temperature (°C)	Analysis Times (Days)
Horse blood	Unpreserved	3	3	20	0, 7, 14, 35, 54, 84
	1% NaF	3	3	20	0, 7, 14, 35, 54, 84
	2% NaF	3	3	20	0, 7, 14, 35, 54, 84
	Unpreserved	3	3	4	0, 7, 14, 35, 54, 84
	1% NaF	3	3	4	0, 7, 14, 35, 54, 84
	2% NaF	3	3	4	0, 7, 14, 35, 54, 84
	Unpreserved	3	3	-18	0, 7, 14, 35, 54, 84
	1% NaF	3	3	-18	0, 7, 14, 35, 54, 84
	2% NaF	3	3	-18	0, 7, 14, 35, 54, 84
Sheep VH	Unpreserved	2	2	20	0, 7, 14, 21, 35, 54, 84
	1.5% NaF	2	2	20	0, 7, 14, 21, 35, 54, 84
	Unpreserved	2	2	4	0, 14, 21, 35, 54, 84
	1.5% NaF	2	2	4	0, 14, 21, 35, 54, 84
	Unpreserved	2	2	-18	0, 14, 21, 35, 54, 84
	1.5% NaF	2	2	-18	0, 14, 21, 35, 54, 84
Deer muscle	Unpreserved (H ₂ O)	4	1	25	0, 7, 14, 28
	2% NaF/H ₂ O	4	1	25	0, 7, 14, 28
	2% NaF/PBS	4	1	25	0, 7, 14, 28
	Unpreserved (H ₂ O)	4	1	4	0, 7, 14, 28
	2% NaF/H ₂ O	4	1	4	0, 7, 14, 28
	2% NaF/PBS	4	1	4	0, 7, 14, 28
	Unpreserved (H ₂ O)	4	1	-18	0, 7, 14, 28
	2% NaF/H ₂ O	4	1	-18	0, 7, 14, 28
	2% NaF/PBS	4	1	-18	0, 7, 14, 28

4.3.2.1 Sample analyses

Matrix matched calibration standards were prepared at concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/L (mg/0.5 kg). Low and high quality control (QC) samples were prepared in the blank matrix at concentrations of 0.03 and 0.3 mg/L (mg/0.5 kg) using independently prepared standard solutions. Negative controls consisted of the blank matrix with no analyte added. Controls were run concurrently with the curves to determine the validity of the calibration. With the exception of blank samples, 100µL of

internal standard (IS) working solution, prepared to give a final concentration of 0.15 mg/L cocaine-d3 and BZE-d3, was added to stability samples, calibration and QC standards. Analyte extraction was performed by SPE with confirmation and quantification by GC-MS/MS. The sample preparation procedure, analytical methods and chemical and reagent information are described in Section 3.9.

Blood and VH extractions and subsequent GC-MS analyses were carried out by Nicola Jones. GC-MS data processing and subsequent statistical analysis and interpretation were carried out independently for the present study.

4.4 Statistical analyses

Graphical representation of data was achieved out using Microsoft Excel 2007. Calculation of the degradation rate kinetic was achieved through regression using SPSS v18. The rate constant for the degradation of cocaine in all matrices was obtained from the slope of the linear regression line relating drug concentration to the time of storage. The difference between degradation rates observed in different preservative and temperature conditions was determined by comparison of regression slopes using the Students *t*-test statistic, computed as the difference between the two slopes divided by the standard error of the difference between the slopes (degrees of freedom = $n - 4$). An alpha probability (p) = .05 demonstrated that the regression slopes, and thus the rates of degradation, were significantly different.

4.5 Results

The percentage loss of cocaine in horse blood, sheep VH and deer muscle at each analysis time and storage condition is given in Table 18. The stability concentration data is presented in Appendix 21 (blood), 22 (VH) and 23 (muscle). Cocaine was considered stable, moderately stable and unstable at individual time points if the respective losses were < 15%, 15 – 30% and >30%. Regression slope comparison data used as a measure of the difference in cocaine degradation rates between selected conditions are displayed in Table 19.

Table 18 Percentage loss of cocaine in horse blood, sheep vitreous and deer muscle following 7 – 84 days storage under different temperature and preservative conditions

Temperature		20°C					4°C					-18°C					
Matrix	Analysis day	7	14	35	54	84	7	14	35	54	84	7	14	35	54	84	
Blood	0% NaF	100	100	100	100	100	100	100	100	100	100	81	100	93	97	100	
	1% NaF	44	97	100	100	100	0	54	63	85	98	0	2	*	3	11	
	2% NaF	48	98	100	100	100	0	34	36	70	88	8	0	*	10	10	
Analysis day		7	14	21	35	54	84	14	21	35	54	84	14	21	35	54	84
Vitreous	0% NaF	87	99	100	100	100	100	76	83	100	100	100	14	24	9	14	28
	1.5 NaF	94	99	100	100	100	100	56	82	88	98	100	13	12	7	17	14
Temperature		25°C			4°C			-18°C			<div>Stable (L < 15%) Moderately stable (L = 15 - 30%) Unstable (L > 30%)</div>						
Analysis day		7	14	31	7	14	31	7	14	31							
Muscle	0% NaF	17	23	78	0	0	2	0	0	0							
	2% NaF	5	7	16	0	0	0	2	0	0							
	2% NaF/PBS	12	37	66	2	0	0	0	0	0							

* Erroneous result obtained for cocaine on day 35 at -18°C for 0% NaF (0.64 mg/L) and 1% NaF (0.62 mg/L)

Table 19 Comparison of regression slopes obtained for cocaine in horse blood, sheep vitreous and deer muscle under different temperature and preservative conditions

Matrix	Temp. (°C)	% NaF	Slope (b)	SE _b	df	<i>t</i> statistic
Horse blood	20	1	0.189	0.021	10	-0.263
		2	0.198	0.027		
	4	1	0.043	0.003	29	4.992*
		2	0.025	0.002		
	20	1	0.189	0.021	21	6.883*
	4	1	0.043	0.003		
	20	2	0.198	0.027	23	6.390*
	4	2	0.025	0.002		
Sheep VH	20	0	0.312	0.016	14	-1.035
		1.5	0.341	0.023		
	4	0	0.084	0.008	19	1.300
		1.5	0.071	0.006		
	20	0	0.312	0.016	13	12.746*
	4	0	0.084	0.008		
	20	1.5	0.341	0.023	20	11.359*
	4	1.5	0.071	0.006		
Deer muscle	25	0	0.972 [†]	0.001	28	6.261*
		2/PBS	0.986 [†]	0.002		

SE_b Standard error of the slope (b); df = degrees of freedom (n-4); * significant *t*-statistic (*p* = .05); [†]Standardised beta coefficient (for comparison of regression with 1st and 0th order kinetics)

4.5.1 Calculation of cocaine degradation kinetics in horse blood, sheep vitreous and deer muscle

When plotting the residual cocaine concentrations against the time of storage in horse blood and sheep VH a non-linear relationship was observed. Plotting the logarithm of concentration as a function of time yielded a linear relationship at each temperature and preservative condition indicating that degradation could be interpreted using first-order kinetics, where the rate of degradation is proportional to the analyte concentration. Rate constants and correlation coefficients as determined from the linear regression slopes are displayed in Table 20. In unpreserved deer tissue homogenates the rate of analyte loss was apparently independent of analyte concentration. A linear relationship was

observed between cocaine concentration and storage time indicating zeroth-order degradation kinetics. Deer tissue samples preserved with NaF and buffered with PBS demonstrated first order kinetics.

Table 20 Rate constant [k (day⁻¹)] and correlation coefficients of cocaine in horse blood, sheep vitreous and deer muscle under different temperature and preservative conditions

Matrix	Temperature (°C)	Preservative Condition (% NaF)	Rate Constant [k (day ⁻¹)]	Correlation coefficient
Horse blood	20	1	0.435	0.919
		2	0.456	0.868
	4	1	0.099	0.935
		2	0.058	0.945
	-18	0	0.129	0.927
Sheep VH	20	0	0.719	0.981
		1.5	0.785	0.968
	4	0	0.193	0.947
		1.5	0.164	0.913
Deer muscle	25	0	0.01*	0.945
		2/PBS	0.083	0.972

*0th-order reaction; cocaine followed 1st order degradation kinetics under all other conditions

4.5.2 pH change in horse blood, sheep vitreous and deer muscle

pH data was only available for days 0, 7, 35 and 84 in horse blood and days 0, 7, 14 and 84 in sheep VH. The pH of unpreserved horse blood rose from its initial value (pH 7.6) on day 0 to pH 7.8 on day 7 in samples stored at 20 and 4°C, and to 7.7 in samples stored at -18°C. By day 35 the pH was 7.6 in samples stored at all temperatures. By the end of the study period the pH was 7.7. In horse blood preserved with NaF, the starting pH was slightly higher (pH 7.7) than in unpreserved blood and had risen to pH 7.9 on day 7 and remained the same on day 35 and 84 following storage at all temperatures.

The initial pH of the sheep VH was 7.6 in unpreserved VH and 7.9 in VH preserved with NaF. In unpreserved samples stored at 20 and 4°C the pH dropped to 7.3 by day 7 and further to 7.0 by day 14. At -18°C the pH remained at 7.9 on days 7 and 14. In

preserved VH the pH increased to 8.0 by day 7 at all temperatures. At the end of the study period the pH in unpreserved and preserved VH stored at all temperatures was pH 7.8 and pH 8.0 respectively.

The pH of unpreserved muscle tissue stored at 25°C changed significantly during the study period, rising from 5.7 at day 0 to 8.2 at day 14 (Figure 11). At 4°C a steep increase was observed by day 7 (pH 6.8) comparable to that observed in samples stored at 25°C with a slight decrease observed thereafter. Unpreserved samples stored at -18°C did not rise above pH 5.9. The addition of NaF stabilised the pH of the tissue samples as effectively as the addition of 1.0 M PBS (pH 6.0). There was no more than a 0.3 pH unit change experienced during the study period in both NaF and buffered/NaF samples stored at any temperature. In whole tissue stored under the temperature conditions investigated in the stability study, the only remarkable pH change was observed at 25°C at the end of the study period, where an increase of 1.3 units had occurred. In whole tissue stored at 4 and -18°C an increase in pH of only 0.1 pH units was observed by day 31. The pH data obtained in muscle tissue under all storage conditions is tabulated in Appendix 24.

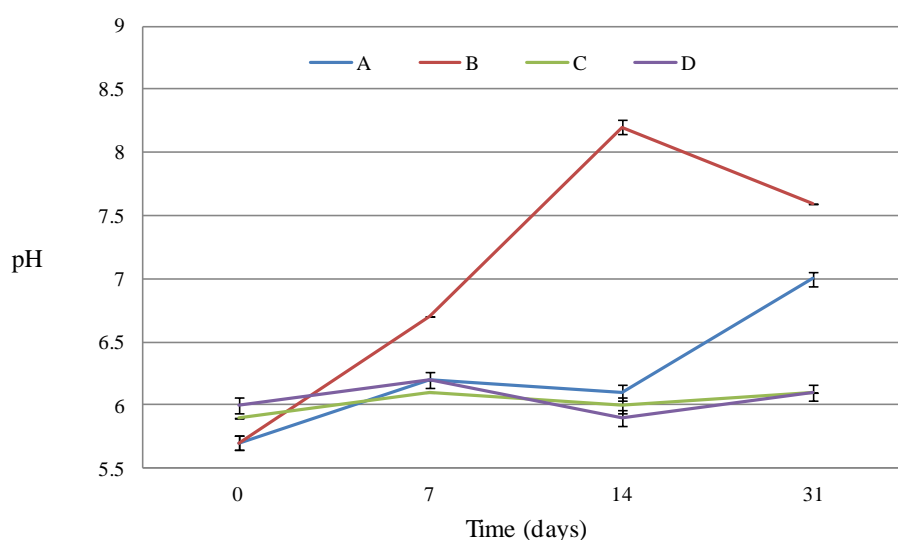


Figure 11 Deer muscle pH change over time in A) whole tissue (25°C); B) tissue + water homogenate (25°C); C) tissue + water/2% NaF homogenate (25°C); D) tissue + 1.0 M phosphate buffer/2% NaF homogenate (25°C), and; E) tissue + water homogenate (4°C).

Error bars = standard deviation

4.5.3 Degradation of cocaine in unpreserved and preserved horse blood

At 20°C and 4°C in unpreserved horse blood cocaine could not be detected at the first analysis time (day 7; Table 18). In unpreserved horse blood stored at -18°C cocaine could not be detected after day 54. BZE profiles in unpreserved samples stored at 20, 4 and -18°C were essentially the same, with low peak concentrations (0.03 – 0.04 mg/L) achieved on day 7 (Figure 12). The concentration of BZE in these samples did not account for more than 9% of the initial cocaine concentration. Samples stored at 20°C with 1 and 2% NaF demonstrated almost identical profiles for cocaine and BZE (Figure 12). Comparison of regression slopes indicated that the degradation rate of cocaine was essentially the same for 1% and 2% NaF ($b = 0.189$ and 0.198 respectively; $p > .05$; Table 19). The cocaine concentration had decreased by 44 and 48% by day 7, and by 97 and 98% on day 14 in the 1% and 2% NaF conditions respectively (Table 18). BZE concentrations were considerably higher in the preserved samples with peak concentrations in both the 1% and 2% NaF samples equating to 64% of the initial cocaine concentration at day 14. At 4°C cocaine could be detected for much longer in preserved samples; the parent drug was still detectable in the 1% NaF (0.008 mg/L) and 2% NaF (0.048 mg/L) samples at the end of the study period. In these samples the peak BZE concentration (observed at day 84) accounted for 55 and 52% of the initial cocaine concentration respectively. At 4°C cocaine stability was noticeably greater in the 2% NaF samples compared with 1% NaF. Comparison of regression slopes at this temperature showed that the rate of degradation was significantly slower in samples stored with 2% NaF ($p = .05$; Table 19). In preserved blood stored at -18°C cocaine was essentially stable for the study period with losses of only 11 and 10% observed in samples stored with 1% and 2% NaF respectively (Table 18). The peak BZE concentration accounted for the total amount of cocaine lost in these samples. In unpreserved samples BZE generally declined after day 7 and could not be detected at the end of the study period in samples stored at any temperature. Following the total loss of cocaine in preserved samples (1% and 2% NaF) stored at 20°C the concentrations of BZE declined from their peak at day 14 by approximately 31% by day 84.

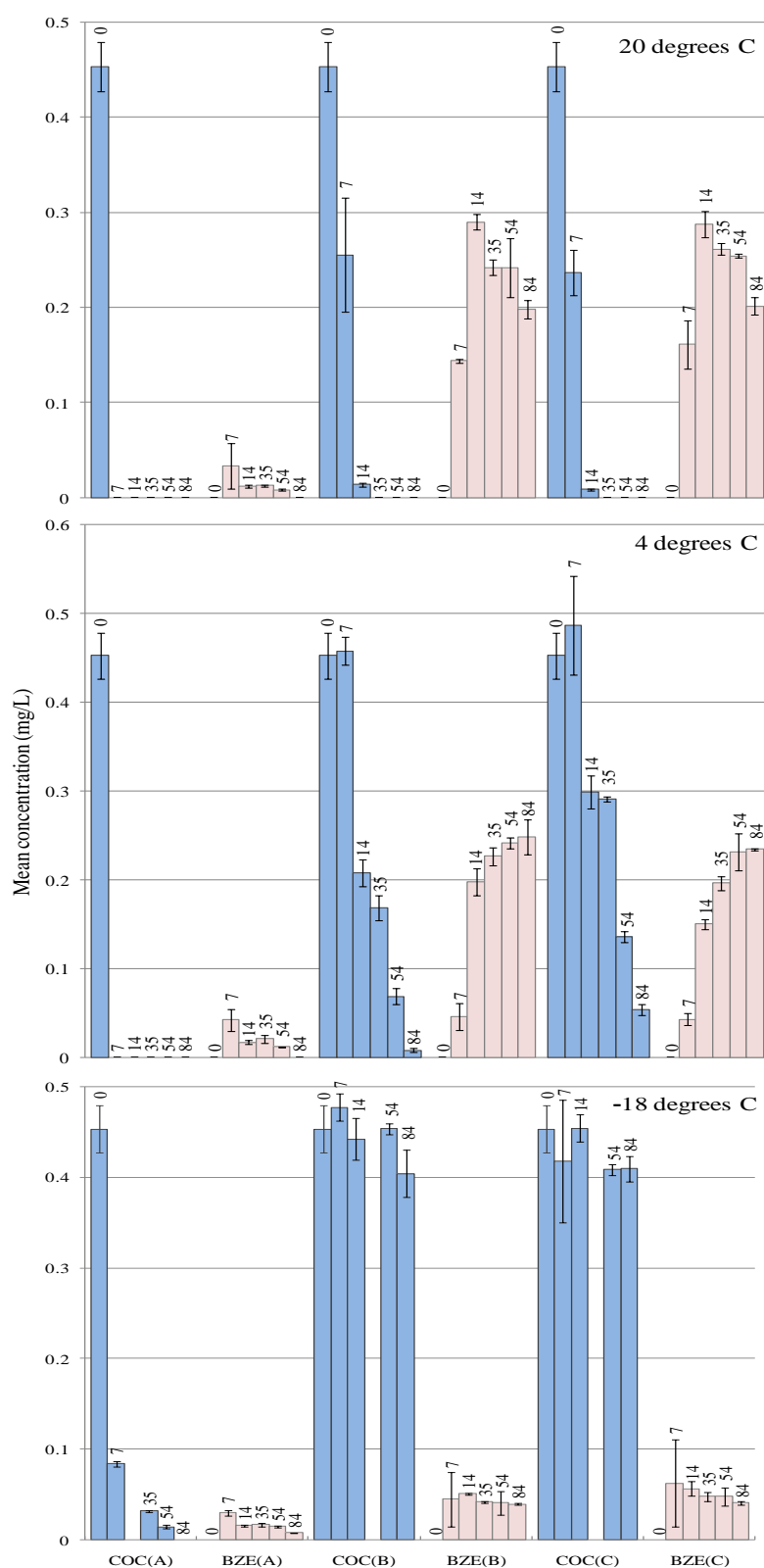


Figure 12 Mean concentrations of cocaine (COC) and BZE in horse blood stored for 84 days at 20, 4 and -18°C with (A) 0% NaF; (B) 1% NaF, and; (C) 2% NaF

Error bars = standard deviation (analysis day next to error bar)

Note: erroneous result obtained for cocaine on day 35 for 0% NaF (0.64 mg/L) and 1% NaF (0.62 mg/L) stored at -18°C (these results are not included on bar chart)

4.5.4 Degradation of cocaine in unpreserved and preserved sheep vitreous

In both preserved sheep VH (PVH) and unpreserved sheep VH (UVH) stored at 20°C cocaine could not be detected by day 21 (Table 18). Comparison of regression slopes showed no significant difference in the rate of degradation of cocaine in PVH and UVH stored at this temperature ($p > .05$). Peak BZE concentrations were reached at day 14 in these samples and accounted for around 79% of the initial cocaine concentration (Figure 13). At 4°C the stability of cocaine increased with 17% still detected in UVH at day 21 (Table 18). In PVH stored at the same temperature cocaine could be detected on day 54 although at a concentration below the LOQ. Despite the extended window of detection for cocaine in PVH stored at 4°C, the rate of degradation was not significantly different to that observed in UVH stored at the same temperature ($p > .05$; Table 19). At this temperature peak BZE concentration was reached at day 35 in UVH and at day 54 in PVH and accounted for approximately 73% of the initial cocaine concentration in both sets of samples (Figure13). Cocaine stability was increased substantially in sheep VH stored at -18°C with recoveries of 86% and 72% observed in PVH and UVH respectively on day 84 (Table 18). BZE accounted for 60 – 81% of the total amount of cocaine lost in these samples (Figure13).

4.5.5 Degradation of cocaine in unpreserved and preserved deer muscle

The concentration of cocaine measured in unpreserved deer muscle (UM), muscle preserved with 2% NaF (PM) and muscle buffered to pH 6.0 and preserved with 2% NaF (BPM) decreased by a maximum of only 2% over the entire study period when stored at 4 and -18°C (Table 18). Given the excellent stability of cocaine at 4 and -18°C cocaine and BZE time concentration profiles are not presented. In UM stored at 25°C cocaine had declined by 17, 23 and 78% on days 7, 14 and 31 respectively (Table 18). A similar pattern was observed in BPM, with losses of 12, 37 and 66% observed on the same days. In PM stored at the same temperature the concentration of cocaine had declined by only 16% by the end of the study period. The amount of BZE measured on day 31 in UM and BPM accounted for the total loss of cocaine (Figure 14).

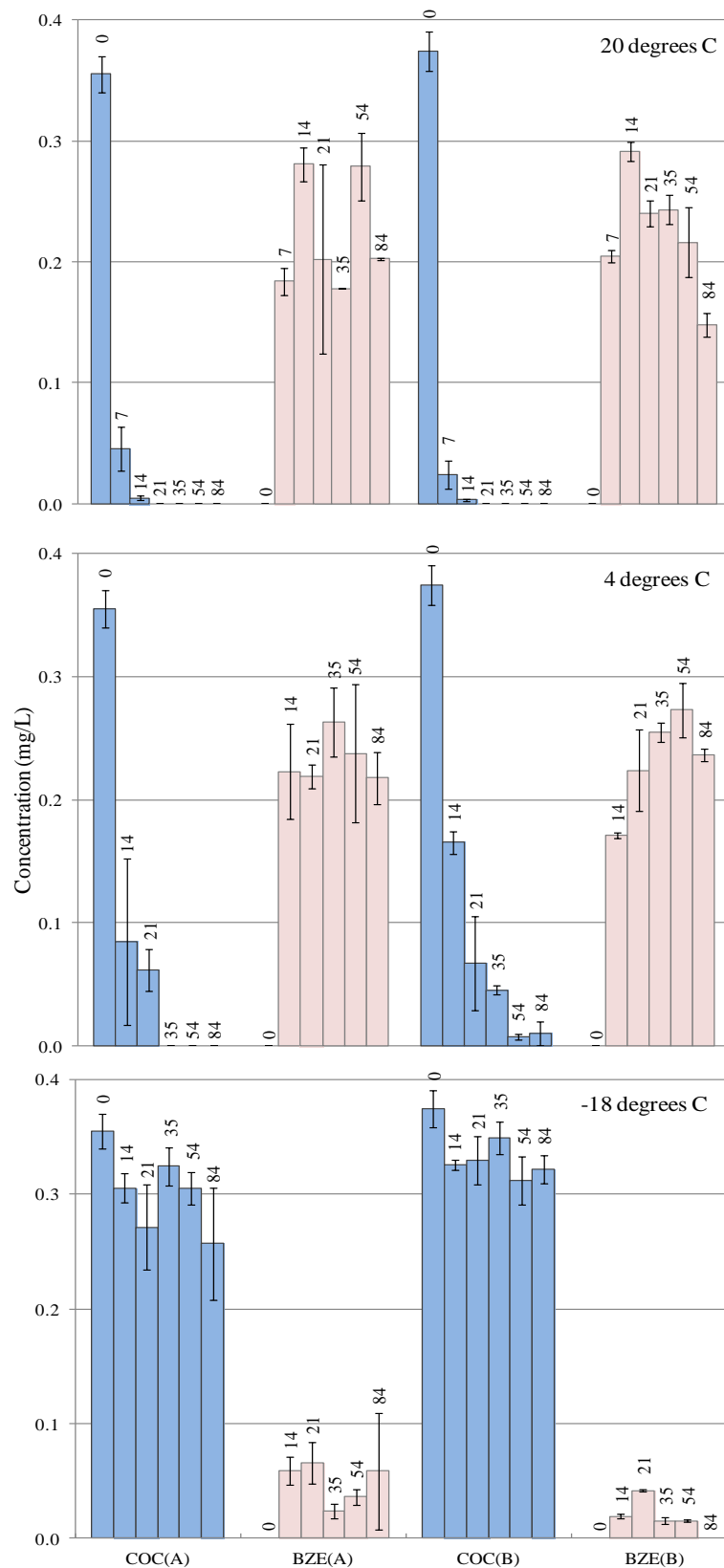


Figure 13 Mean concentrations of cocaine (COC) and BZE in sheep vitreous stored for 84 days at 20, 4 and -18°C with (A) 0% NaF, and; (B) 1.5% NaF

Error bars = standard deviation (analysis day next to error bar)

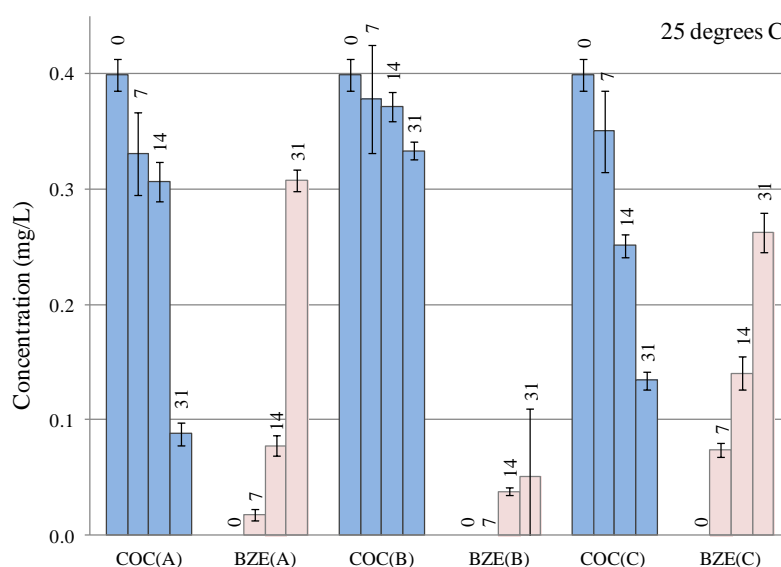


Figure 14 Mean concentrations of cocaine (COC) and BZE in deer muscle homogenised with (A) water; (B) water+2% NaF, and; (C) buffer+2% NaF stored at 25°C for 31 days

Error bars = standard deviation (analysis day next to error bar)

4.6 Discussion

Cocaine was highly unstable in unpreserved horse blood with 100% loss observed by day 7 in samples stored at 20 and 4°C. Even with storage at -18°C only 19% of the initial concentration remained on day 7 with none detected by the end of the study period. These results are in contrast to a previous report on unpreserved fresh human blood in which 50% of the original cocaine concentration could be detected 2 weeks following storage at 4°C and 75% detected 110 days following storage at -15°C (Isenschmid et al. 1989). As with human plasma, horse plasma also contains BuChE (Aldridge 1953) which hydrolyses cocaine to EME. The apparent decrease in cocaine stability in horse blood as compared with human blood might be attributed to the presence of high levels of carboxylesterase (CE) in horse plasma (Li et al. 2005). Humans express two types of CE (hCE-1 and hCE-2) both predominantly in the liver (Satoh et al. 2002) and minimally in white blood cells (Saboori and Newcombe 1990; Munger et al. 1991). hCE-1 catalyses the formation of BZE whereas hCE-2, like BuChE, converts cocaine to EME (Dean et al. 1991; Kamendulis et al. 1996; Pindel et al. 1997). Isenschmid et al. (1989) observed cocaine to hydrolyse more readily to EME in the presence of CE (from porcine liver) than in the presence of BuChE (from horse plasma). Very little BZE was detected in unpreserved horse blood in this study implying

that enzyme hydrolysis forming EME (not analysed) was the major pathway for cocaine degradation. The results are consistent with previous reports that in unpreserved human blood EME is formed almost exclusively (Isenschmid et al. 1989). Although the level of expression of specific CEs in horse blood could not be determined from the literature, the rapid decline of cocaine in unpreserved samples and the very low associated BZE concentrations might suggest that the predominant CE present in horse blood is more comparable to hCE-2 and thus preferential EME formation.

Although the addition of NaF increased the stability of cocaine substantially in horse blood, the observed stability appeared to be less than that has previously reported for preserved human blood. Baselt et al. (1983) reported that in human blood preserved with 0.5% NaF and stored at 4°C, 80% of the original concentration (1.0 mg/L) could still be detected after 21 days (Baselt 1983). In horse blood preserved with 1% NaF and stored at the same temperature, only 46% of the original cocaine concentration (0.4 mg/L) could be detected after 14 days and none could be detected on day 35. Isenschmid et al. (1989) observed a 50% loss of cocaine in refrigerated human blood preserved with 2% NaF over a 110 day study period. In the present study only 10% cocaine remained on day 84 in horse blood stored under the same conditions. These results further reflect the influence of the additional hydrolytic pathway provided by CE in horse blood. This has significant implications for toxicology laboratories that store calibration and/or quality control standards prepared in this matrix.

Cocaine stability as a function of storage temperature was well demonstrated in preserved horse blood. Stability increased substantially as the storage temperature decreased. The rate of degradation was found to be significantly slower in blood stored at 4°C compared to at 20°C ($p = .05$). At 20 and 4°C the amount of BZE measured in preserved samples was much higher than in unpreserved samples. This observation has been reported previously in human blood (Baselt 1983; Isenschmid et al. 1989) and may be attributed to the inhibition of esterase mediated hydrolysis and subsequent shunting of the cocaine metabolic pathway to chemical hydrolysis. With the exception of preserved horse blood stored at -18°C, the BZE concentration typically accounted for around half the initial cocaine concentration indicating that enzymatic hydrolysis did not cease completely *in vitro* with either concentration of NaF. The ability of NaF to only partially inhibit esterase activity has been reported previously (Stewart et al. 1977).

The influence of NaF concentration on cocaine stability in horse blood was noticeable only at 4°C where cocaine loss observed in the 1% condition exceeded that in the 2% condition by $\geq 15\%$ through days 14 to 54. From comparison of regression slopes the rate of degradation was found to be significantly slower in samples stored with 2% ($b = 0.025$) compared with 1% NaF ($b = 0.043$; $p = .05$). At 20°C cocaine could not be detected in either preservative condition following day 14 and the rate of degradation was not statistically different ($p > .05$). The rapid decline of cocaine at 20°C irrespective of NaF concentration can partly be attributed to the acceleration of chemical hydrolysis with increasing temperature (Isenschmid et al. 1989; Skopp et al. 2001a). BZE concentrations accounted for 64% of the initial cocaine concentration in both 1% and 2% conditions at 20°C. Preserved horse blood stored at -18°C yielded approximately 90% of the original cocaine concentration at the end of the study period. This observation is more comparable to previous reports for human blood stored in the same manner and well reflects the effectiveness of the combination of freezing and NaF addition in stabilising horse and human blood samples for cocaine analysis.

In unpreserved horse blood stored at all temperatures and in preserved horse blood stored at 20°C BZE concentrations generally declined following the total loss of cocaine. In unpreserved samples, BZE, which peaked at day 7 (0.03 – 0.042 mg/L), could no longer be detected at the end of the study period following storage at any temperature. By day 84 in preserved samples stored at 20°C the concentration of BZE declined from its peak at day 14 by approximately 31% in the 1% and 2% NaF conditions. In human blood BZE is known to undergo further hydrolysis forming ECG (Skopp et al. 2001a). Since BZE retains the benzoyl ester function this hydrolysis is thought to be enzyme mediated and as with the hydrolysis of cocaine to EME, is catalysed by BuChE (Stewart et al. 1977; Skopp et al. 2001a). In horse blood this reaction could also be mediated by CE. The decline in BZE concentration that was observed in this study was certainly far more gradual in comparison to that of cocaine. This observation has been previously reported and has been attributed to BZE having lower affinity for BuChE compared to cocaine (Smirnow and Logan 1996). In preserved horse blood stored at 4°C a significant decline in cocaine concentration through days 35 to 84 was accompanied by a relatively consistent BZE concentration (Figure 12). This observation might be attributed to the competing processes of spontaneous BZE formation and enzyme mediated loss.

The results obtained in this study highlight the importance of NaF addition to horse blood in the preparation of calibration and check standards, particularly if these standards are prepared in bulk and stored prior to use. Even with frozen storage, enzyme activity continues to a significant degree in the absence of NaF. This is clearly reflected by the 81% decline in cocaine concentration observed by day 7 in frozen unpreserved horse blood. With the addition of 1% or 2% NaF, cocaine was found to be stable (0% loss) in horse blood for 7 days at 4°C and for the entire study period when stored at -18°C.

The stability of cocaine in sheep VH was noticeably greater than that observed in unpreserved horse blood and was predominantly dependent on storage temperature. Comparison of regression slopes showed that the rate of cocaine degradation in unpreserved sheep VH (UVH) and preserved sheep VH (PVH) stored at 20°C was significantly faster than in the corresponding condition stored at 4°C ($p = .05$). Nevertheless cocaine could not be detected by the end of the study period in any samples stored at 20 or 4°C. Although at 4°C the detection window for cocaine in PVH (54 days) was longer than in UVH (21 days) and the % loss observed at day 7 was greater for UVH (76%) than for PVH (56%), the rates of degradation were not significantly different ($p > .05$). The rate of degradation in UVH and PVH at 20°C was also not different. At -18°C the pattern of loss was similar in UVH and PVH although at the end of the study period UVH demonstrated greater loss (28%) compared with PVH (14%). The unremarkable effect of NaF on the stability of cocaine in sheep VH is in contrast to results obtained for 6AM, where the addition of NaF to sheep VH was shown to significantly increase stability at 20, 4 and -18°C (Chapter 5).

Given the lack of esterases present in VH, the rapid disappearance of cocaine in this matrix may be attributed to spontaneous chemical hydrolysis. The pH of the sheep VH used in this study ranged from 7.0 to 7.6 in unpreserved VH and 7.9 to 8.0 in preserved VH during the first 14 days. By the end of the study period the pH was 7.8 and 8.0 in unpreserved and preserved VH respectively. Within this pH range cocaine would be expected to undergo fairly rapid spontaneous chemical hydrolysis forming BZE (Isenschmid et al. 1989; Kiszka et al. 2000; Romolo et al. 2003). The amount of cocaine loss observed in UVH and PVH at 7 – 21 days storage at 20°C was comparable to that observed in buffered solutions of pH 7.4 – 8.0 stored at 25°C for similar time

periods (Kiszka et al. 2000). In this study BZE accounted for approximately 75% of the initial cocaine concentration in sheep VH samples, supporting the notion that chemical hydrolysis was the predominating mechanism for cocaine breakdown. The fact that a non-stoichiometric relationship was observed in the sheep VH indicates the formation of additional products in this matrix, such as benzoic acid and methyl benzoate, which are also formed from cocaine hydrolysis (Garrett and Seyda 1983; Dejarne et al. 1997). In buffered solution a stoichiometric relationship between the concentration of cocaine and BZE has been observed (Kiszka et al. 2000).

The pH of sheep VH as measured in this study was higher than that typically observed for post-mortem human VH. In 40 cases of varying post-mortem interval the pH measured in human VH was approximately 7.0 (Antonides et al. 2007). The stability of cocaine in human VH during storage might therefore be greater than that observed in this study.

In contrast to horse blood and sheep VH, cocaine was remarkably stable in deer muscle tissue stored at 4°C and below with a maximum loss of only 2% observed at any time point. The increased stability may be largely attributed to the low pH of the muscle homogenates; the pH was 5.7, 5.9 and 6.0 in unpreserved deer muscle (UM), preserved deer muscle (PM) and buffered preserved deer muscle (BPM) respectively. The pH did not increase by more than 0.3 units except for in UM stored at 4°C where the pH rose to 6.8 by day 7. The pH of human and animal skeletal muscle *in vivo* is reported to be approximately pH 7.0 (Pan et al. 1988; Yoshioka et al. 2002; Lawrie and Ledward 2006). As a consequence of post-mortem metabolism and conversion of glycogen to lactic acid, muscle pH has been reported (in cattle) to drop below pH 6.0 within hours after death (Lawrie and Ledward 2006). The lack of esterase activity combined with the low pH would effectively inhibit the spontaneous chemical hydrolysis of cocaine. This finding would also be expected in human muscle given that early post-mortem pH values ranging from pH 5.7 – 6.0 have been reported (Langford et al. 1998).

At 25°C the pH of UM rose from 5.7 at the start of the study to 8.2 by day 14 and then decreased to pH 7.6 at day 31. Given the steep increase in pH observed in UM, the significant decline in cocaine observed by day 31 (78%) was not unexpected. Nevertheless, the stability of cocaine in UM stored at 25°C greatly exceeded that

observed in unpreserved horse blood and sheep VH stored at 4°C. These results are supported by those of Moriya and Hashimoto (1996), who compared the *in vitro* stability of cocaine in blood and femoral muscle obtained from cocaine administered rabbits. Following 5 days storage at 20 – 25°C the authors found $26.2 \pm 19.4\%$ of the original cocaine was still detectable in muscle, whereas in blood cocaine was undetectable within 1 day.

The amount of BZE measured on day 31 accounted for the total loss of cocaine in UM stored at 25°C. Given the lack of hydrolytic esterase activity in skeletal muscle tissue the BZE can be solely attributed to the spontaneous chemical hydrolysis of cocaine catalysed by the high pH of the sample. The rate of tissue putrefaction in UM was clearly accelerated by the addition of water. In intact deer tissue samples stored at 25°C the pH change was less than that observed in homogenised deer tissue (unpreserved), increasing from 5.7 on day 0 to a maximum pH of 7.0 on day 31 (Figure 11).

The addition of 2% NaF to water homogenates stabilised the pH of the deer tissue as effectively as the addition of 1.0 M PBS (pH 6.0) containing 2% NaF. In PM and BPM the starting pH of the tissues were 5.9 and 6.0 respectively and neither sample pH rose by more than 0.2 units. Although minimal increase in pH was observed in both PM and BPM, the stability of cocaine in BPM was much less than that observed in PM. In PM the concentration of cocaine had declined by only 16% by the end of the study period, whereas in BPM the loss of cocaine was 66%, not remarkably less than that observed in UM (78%). Since the pH was comparable in both conditions greater chemical hydrolysis would not necessarily be expected. The greater instability of cocaine observed in BPM might be attributed to the presence of phosphate buffer ions. Gupta (1982) reported that the hydrolysis of cocaine in buffered solutions was catalysed by HPO_4^{2-} and OH^- at pH levels above 5.5. Surprisingly, comparison of regression slopes showed the rate of degradation in BPM to be significantly more rapid than in UM. The degradation in BPM followed 1st order reaction kinetics consistent with that observed in horse blood and sheep VH. In contrast cocaine degradation in unpreserved deer muscle followed zeroth-order kinetics and thus was not proportional to initial cocaine concentration. The change in the reaction kinetic might be attributed to the steep increase in pH observed in the unpreserved deer muscle over the study period.

The stability of cocaine in buffered preserved deer muscle was of particular interest because human tissue samples obtained from authentic cases during this research study were homogenised with PBS fortified with 2% NaF and stored at -18°C in the event re-analysis was required. To assess the stability of cocaine in real samples, 14 homogenates prepared from the thigh muscle of a single individual were re-analysed after 13 months of storage. On average the cocaine concentration had declined by only 15% (range: 5 – 25%; SD: 4.4) reflecting the effectiveness of frozen storage in ensuring the long term stability of cocaine even in the presence of phosphate buffer ions which, in deer muscle (buffer preserved) stored at 25°C, appear to catalyse the hydrolysis of cocaine.

4.7 Conclusion

This study has demonstrated that cocaine is stable for only 7 days in refrigerated horse blood fortified with $\geq 1\%$ NaF. The stability of cocaine in this matrix appeared to be substantially less than that previously reported in the literature for human blood. This finding is of importance for laboratories in which calibration and/or control standards prepared in this matrix are stored prior to use. To ensure the long-term stability of cocaine in horse blood, the addition of sodium fluoride combined with frozen storage is vital. The addition of NaF to sheep VH samples did not remarkably increase the stability cocaine in this matrix whereas frozen storage was found to be essential. The stability of cocaine in deer muscle tissue homogenates greatly exceeded that in horse blood and sheep VH at every temperature analysed irrespective of NaF addition. The increased stability of cocaine in deer muscle can be attributed to low post-mortem pH as well as lack of esterase activity, features that are comparable in human muscle. These findings promote the use of human muscle as a toxicological specimen in which cocaine may be detected for longer compared with blood or VH, providing that tissue samples that cannot be analysed promptly are stored at 4°C or below.

Chapter 5.0 The stability of 6-acetylmorphine in horse blood, sheep vitreous and homogenised deer muscle with and without the addition of sodium fluoride

5.1 Abstract

This study examined the *in vitro* stability of 6-acetylmorphine (6AM) in horse blood, sheep vitreous humour (VH) and homogenised deer muscle stored with and without sodium fluoride (NaF) at three temperatures. Blood and VH were stored with and without NaF at 20, 4 and -18°C for 84 days. The effect of NaF concentration (1% and 2%) was determined for blood. Muscle tissue homogenates were prepared in water with and without NaF and also in phosphate buffer (pH 6.0) containing NaF and stored for 31 days at 25, 4 and -18°C. 6AM and morphine were extracted using SPE and quantified by GC-ion trap-MS/MS. In the absence of NaF, 6AM could not be detected after 7 and 14 days in horse blood stored at 20 and 4°C respectively. At -18°C 6AM was stable for 7 days (12% loss) with 54% still detected on day 84. At every temperature analysed the stability of 6AM in preserved blood was substantially higher than in unpreserved blood. The rate of degradation was found to be significantly slower in blood preserved with 2% NaF compared with 1% NaF ($p = .05$). Irrespective of NaF concentration 6AM was stable for the entire period in blood stored at -18°C. The addition of NaF to VH was essential in stabilising 6AM. On day 7, 6AM had declined by 61, 52 and 42% in VH stored at 20, 4 and -18°C respectively. In preserved samples the concentration had declined by 22% on day 7 following storage at 20°C with 0% loss observed in VH stored at 4 and -18°C at the same time. 6AM was relatively stable in muscle tissue stored with preservative at 4°C and in all samples stored at -18°C. At 20°C 6AM degraded more quickly, particularly in unpreserved tissue. In general the stability of 6AM observed in both preserved and unpreserved muscle tissue exceeded that observed in blood and VH.

5.2 Introduction

The detection of the unique heroin metabolite, 6AM, provides unequivocal evidence of heroin use (Meadway et al. 1998) and thus its detection in biological samples is of great importance. Very few studies have been reported regarding the *in vitro* stability of 6AM in human blood (Pichini et al. 1999; Boy et al. 2008) and none have been reported in horse blood. To date the only information available regarding the effect of preservative on 6AM stability in blood is that provided by Wathanafa and Cooper (2010) who reported that the addition of NaF slowed but did not prevent the breakdown of 6AM in human blood stored at 4°C. Carboxylesterase, which is present in horse blood (Aldridge 1953), hydrolyses 6AM to morphine (Kamendulis et al. 1996; Pindel et al. 1997) and so knowledge of the stability of 6AM in horse blood is of importance if calibration and check standards are prepared in this matrix and stored prior to use. Although the principal value of VH in 6AM determinations is reported to be the extended window of detection it provides compared with blood (Pragst et al. 1999; Antonides et al. 2007), no studies have systematically investigated the *in vitro* stability of this forensically important metabolite in VH. There is currently no information pertaining to the stability of 6AM in muscle tissue.

This study examined the effect of storage temperature and sodium fluoride addition on 6AM stability in horse blood, VH and muscle tissue homogenates. The study period spanned 84 days for blood and VH and 31 days for muscle tissue. Stability was assessed over time by measurement of 6AM and its hydrolysis product, morphine.

5.3 Methods

Horse blood and sheep VH samples were spiked concurrently with cocaine (Chapter 4) to achieve a final 6AM concentration of 0.15 mg/L. This concentration was selected as it represents the higher end of the range of 6AM concentrations typically observed in forensic cases (Chapter 7; Goldberger et al. 1994; Wyman and Bultman 1997; Pragst et al. 1999). The study design was as described earlier for cocaine (Chapter 4). Briefly, samples were analysed in triplicate at three preservative conditions (0%, 1% and 2% NaF) and at three different temperatures (20, 4 and -18°C). VH aliquots (1.5 mL) were prepared in duplicate at two preservative conditions (0 and 1.5% NaF) stored at the same temperatures. Deer muscle tissue homogenates were prepared with 1) water, 2)

water containing 2% NaF, and 3) 1.0 M phosphate buffer (pH 6.0) containing 2% NaF. Homogenate samples (2g; equivalent to 0.5 g tissue) were spiked with 150 ng 6AM and set up in quadruplet at each storage condition. Storage conditions and analysis time for each matrix are presented in Table 17.

5.3.1 Sample analyses

Matrix matched calibration standards were prepared at concentrations of 0.005, 0.0075, 0.01, 0.025, 0.05, 0.1 and 0.2 mg/L (mg/0.5 kg) for 6AM and 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/L (mg/0.5 kg) for morphine. Low and high quality control (QC) samples were prepared in the blank matrix at concentrations of 0.0125 and 0.04 mg/L (mg/0.5 kg) for 6AM and 0.03 and 0.3 mg/L (mg/0.5 kg) for morphine using independently prepared standard solutions. Negative controls consisted of the blank matrix with no analyte added. Controls were run concurrently with the curves to determine the validity of the calibration. With the exception of blank samples, 100µL of internal standard (IS) working solution, prepared to give a final concentration of 0.04 mg/L 6AM-d3 and 0.15 mg/L morphine-d3, was added to stability samples, calibration and QC standards. Analyte extraction was performed by SPE with confirmation and quantification by GC-MS/MS. The sample preparation procedure, analytical methods and chemical and reagent information are described in Section 3.9.

5.4 Statistical analysis

Statistical analyses are detailed in Section 4.4.

5.5 Results

The percentage loss of 6AM in horse blood, sheep VH and deer muscle at each analysis time and storage condition is given in Table 21. The stability concentration data is presented in Appendix 25 (horse blood), 26 (sheep VH) and 27 (deer muscle). 6AM was considered stable, moderately stable and unstable at individual time points if the respective losses were < 15%, 15 – 30% and > 30%. Regression slope comparison data used as a measure of the difference in 6AM degradation rates between selected conditions are displayed in Table 22.

Table 21 Percentage loss of 6AM in horse blood, sheep vitreous and deer muscle following 7 – 84 days storage under different temperature and preservative conditions

Temperature		20°C					4°C					-18°C					
Matrix	Analysis day	7	14	35	54	84	7	14	35	54	84	7	14	35	54	84	
Blood	0% NaF	89	100	100	100	100	54	92	100	100	100	12	36	33	45	46	
	1% NaF	31	59	84	91	98	15	16	39	54	71	7	0	0	4	8	
	2% NaF	23	49	73	86	95	13	0	15	36	46	17	0	0	2	7	
Analysis day		7	14	21	35	54	84	14	21	35	54	84	14	21	35	54	84
Vitreous	0% NaF	61	80	93	100	100	100	52	60	89	93	100	42	54	48	68	95
	1.5 NaF	22	31	53	78	90	97	0	6	4	19	27	0	4	10	4	10
Temperature		25°C			4°C			-18°C									
Analysis day		7	14	31	7	14	31	7	14	31							
Muscle	0% NaF	21	57	100	14	19	31	4	8	16	Stable (L < 15%)						
	2% NaF	11	23	35	7	16	20	8	16	21	Moderately stable (L = 15 - 30%)						
	2% NaF/PBS	14	30	46	3	13	24	9	5	19	Unstable (L > 30%)						

Table 22 Comparison of regression slopes obtained for 6AM in horse blood, sheep vitreous and deer muscle stored under different temperatures and preservative conditions

Matrix	Temp. (°C)	% NaF	Slope (b)	SE _b	df	<i>t</i> statistic
Horse blood	20	1	0.043	0.001	30	5.657*
		2	0.035	0.001		
	4	1	0.015	0.00	31	7.0*
		2	0.008	0.001		
Sheep VH	20	0	0.106	0.007	23	8.791*
		1.5	0.042	0.002		
	4	0	0.052	0.005	24	9.414*
		1.5	0.004	0.001		
	20	1.5	0.042	0.002	24	1.857*
	4	0	0.052	0.005		
Deer muscle	20	0	0.953 [†]	0.001	24	5.814*
		2/PBS	0.940 [†]	0.002		

SE_b Standard error of the slope (b); df = degrees of freedom (n-4); * significant *t*-statistic ($p = .05$); [†] Standardised beta coefficient (for comparison of regression with 1st and 0th order kinetics)

5.5.1 Calculation of 6AM degradation kinetics in horse blood, sheep vitreous and deer muscle

When plotting the residual 6AM concentrations against the time of storage in horse blood and sheep VH a non-linear relationship was observed. Plotting the logarithm of concentration as a function of time yielded a linear relationship at each temperature and preservative condition indicating that degradation could be interpreted using first-order kinetics, where the rate of degradation is proportional to the analyte concentration. Rate constants and correlation coefficients as determined from the linear regression slopes are displayed in Table 23. In unpreserved deer tissue homogenates the rate of analyte loss was, as with cocaine, apparently independent of analyte concentration. A linear relationship was observed between 6AM concentration and storage time indicating zeroth-order degradation kinetics. Deer tissue samples preserved with NaF and buffered with PBS demonstrated first order kinetics.

Table 23 Rate constant [k (day⁻¹)] and correlation coefficients of 6AM in horse blood, sheep vitreous and deer muscle stored under different temperature and preservative conditions

Matrix	Temperature (°C)	% NaF	Rate constant [k (day ⁻¹)]	Correlation coefficient
Horse blood	20	0	0.716	0.996
		1	0.099	0.990
		2	0.081	0.996
	4	0	0.410	0.955
		1	0.035	0.989
		2	0.018	0.866
Sheep VH	20	0	0.244	0.967
		1.5	0.097	0.979
	4	0	0.120	0.931
	-18	0	0.067	0.856
	20	0	0.008*	0.965
		2/PBS	0.046	0.883

*0th order reaction; 6AM followed 1st order degradation kinetics under all other conditions

5.5.2 pH change in horse blood, sheep vitreous and deer muscle tissue

The pH change observed in horse blood, sheep VH and deer muscle has been reported in Section 4.5.2. The pH change over time in deer tissue is illustrated in Figure 11 and tabulated in Appendix 24.

5.5.3 Degradation of 6AM in unpreserved and preserved horse blood

In unpreserved horse blood the stability of 6AM increased as storage temperature decreased. At 20°C 6AM had decreased by 89% at day 7 and was not detected on day 14 (Table 21). At 4°C the stability was increased with just over half of the initial concentration detected at day 7 and 8% remaining on day 14. The peak morphine concentration measured at both 20°C and 4°C in unpreserved horse blood accounted for approximately 50% of the initial 6AM concentration (Figure 15). At -18°C 6AM was stable in unpreserved blood for 7 days (loss = 12%) and 54% could be detected on day 84. The morphine concentration measured on day 84 accounted for 50% of the amount of 6AM loss (Figure 15). With the addition of NaF the stability of 6AM in horse blood was increased substantially within each temperature condition. In preserved horse blood

stored at -18°C 6AM was stable with a maximum loss of only 8% observed by the end of the study period (Table 21). At 20°C the 6AM and morphine concentration profiles were visually very similar for blood preserved with 1% and 2% NaF (Figure 15). However, comparison of regression slopes indicated that the degradation rate of 6AM in the 2% samples ($b = 0.043$) was significantly slower compared with the 1% samples ($b = 0.035$; $p = .05$; Table 22). At 4°C the difference in 6AM stability in the 1% and 2% NaF conditions was even more apparent with 29% and 54% of the initial 6AM concentration detected on day 84 respectively. The rate of degradation was found to be significantly slower in the 2% ($b = 0.008$) compared to 1% ($b = 0.015$) NaF condition ($p = .05$; Table 22). At 20°C the morphine concentration measured in 1 and 2% PB at the end of the study period respectively accounted for 54 and 59% of the amount of 6AM lost. At 4°C these values were 60 and 75% respectively. 6AM was essentially stable for the entire study period in PB stored at -18°C (Table 21; Figure 15).

5.5.4 Degradation of 6AM in unpreserved and preserved sheep vitreous

In unpreserved sheep VH (UVH) samples the stability of 6AM increased as storage temperature decreased. On analysis day 7 the concentration of 6AM had declined by 61, 52 and 42% in UVH stored at 20, 4 and -18°C respectively (Table 21). At 20 and 4°C 6AM could not be detected after 21 and 54 days respectively. At -18°C 6AM was detected in UVH at the end of the study period although the concentration had decreased by 95% of that initially measured. The peak morphine concentration accounted for between 56 and 66% of the initial 6AM concentrations in UVH (Figure 16). The addition of 1.5% NaF to VH had a significant effect on 6AM stability at each temperature studied. In preserved samples (PVH) stored at 20°C, 78% of the initial 6AM concentration was present on day 7 and the drug was still detectable at the end of the study period. At 4°C 6AM was stable (loss < 15%) up to day 35 with only a moderate decline (27%) by day 84. Comparison of regression slopes showed that the rate of degradation observed at 20 and 4°C was significantly slower in PVH compared with UVH ($p = .05$; Table 22). In PVH stored at -18°C 6AM was essentially stable for the duration of the study period with a maximum loss of only 10% by day 84. With the exception of samples stored at 20°C the amount of morphine measured in PVH at the end of the study period accounted for the observed decline in 6AM (Figure 16). At 20°C morphine accounted for approximately 50% of the initial 6AM concentration (Figure 16).

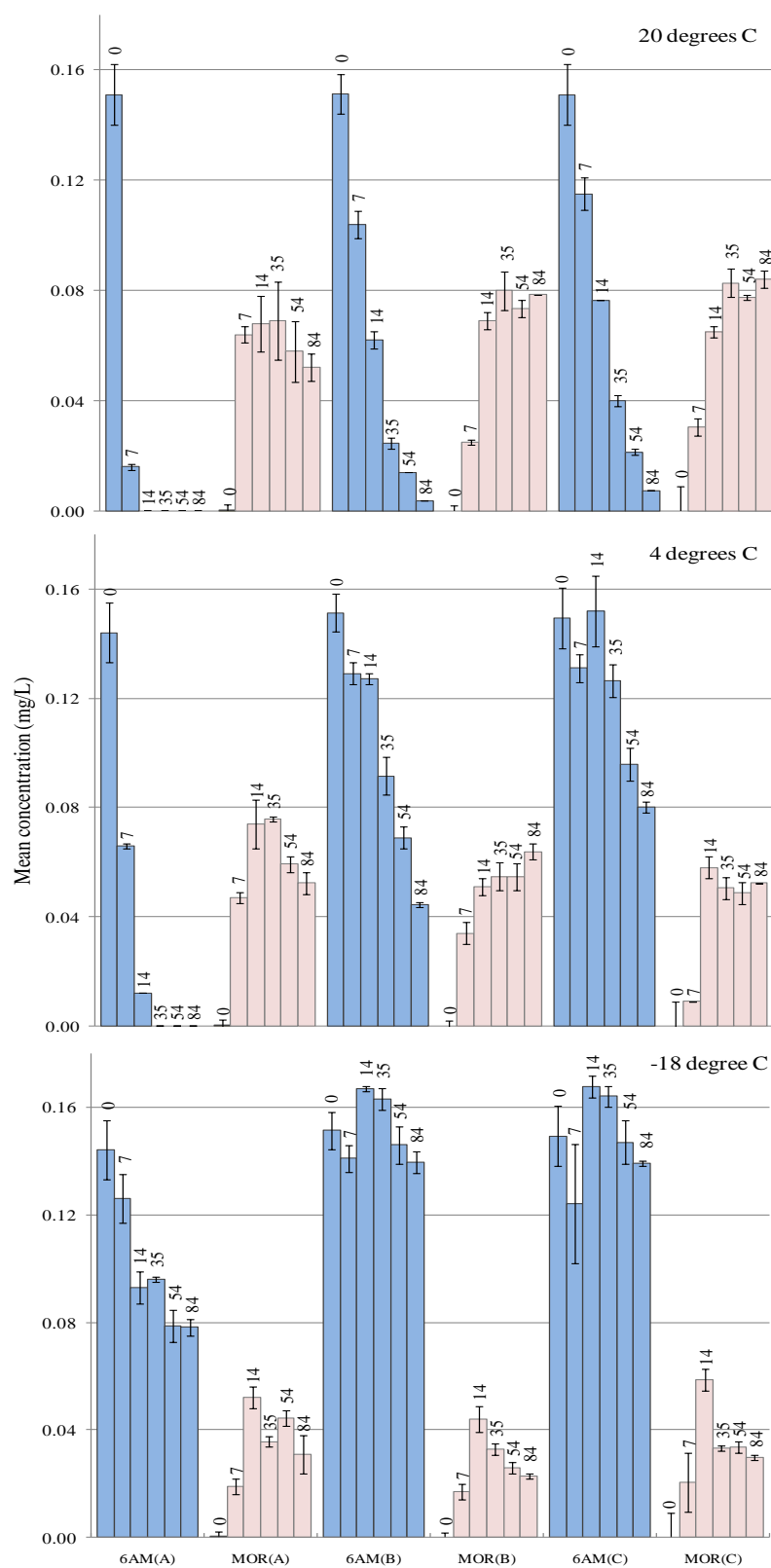


Figure 15 Mean concentrations of 6AM and morphine (MOR) in horse blood stored for 84 days at 20, 4 and -18°C with (A) 0% NaF; (B) 1% NaF, and; (C) 2% NaF

Error bars = standard deviation (analysis day next to error bar)

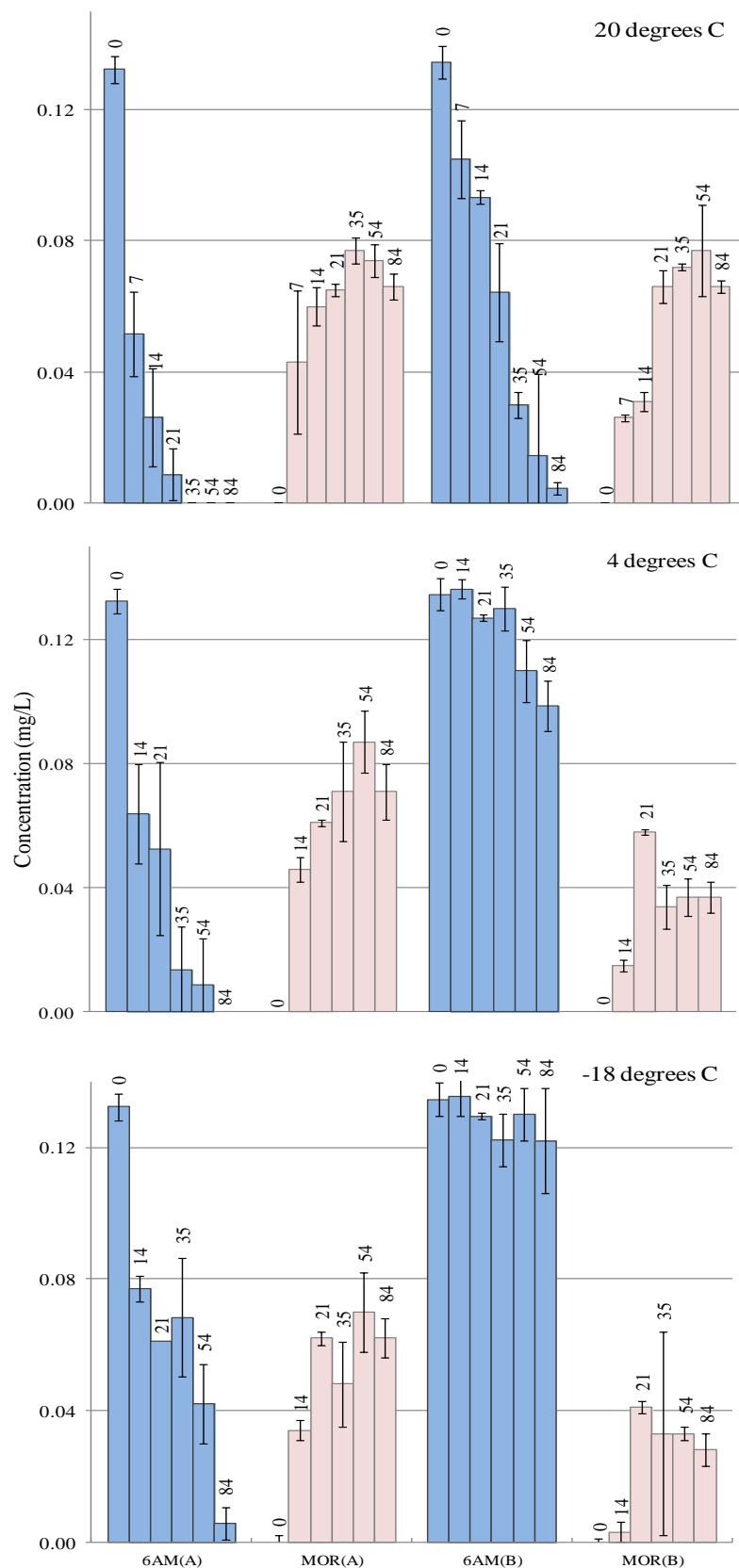


Figure 16 Mean concentrations of 6AM and morphine (MOR) in sheep vitreous stored for 84 days at 20, 4 and -18°C with (A) 0% NaF, and; (B) 1.5% NaF

Error bars = standard deviation (analysis day next to error bar)

5.5.5 Degradation of 6AM in unpreserved and preserved deer muscle

In unpreserved deer muscle (UM) the stability of 6AM increased with decreasing storage temperature. At 25°C 6AM could no longer be detected in UM by the end of the study period and at 4 and -18°C 6AM had declined by 31 and 16% respectively (Table 21). 6AM was found to be stable for 7 days in deer muscle preserved with 2% NaF (PM) and in buffered preserved deer muscle (BPM) stored at all temperatures (loss \leq 14%). At 25°C losses of 35 and 46% were observed for PM and BPM respectively by day 31. At 4 and -18°C the stability profile of 6AM in PM and BPM was not remarkably different (Figure 17). In general the concentration of morphine increased as 6AM declined but the relationship at individual time points was rarely stoichiometric. In samples in which 6AM was unstable (loss > 30%), morphine accounted for between 36 and 86% of the 6AM lost by the end of the study period (Figure 17).

5.6 Discussion

In unpreserved horse blood stored at 20 and 4°C 6AM was highly unstable and could not be detected following day 7 and 14 respectively. Storage at -18°C reduced the rate of decline substantially with 54% still detectable at the end of the study period. In humans the enzyme mediated hydrolysis of 6AM in blood is catalysed only by erythrocyte AChE (Salmon et al. 1999). In the liver this reaction is catalysed by carboxylesterase (CE) (Kamendulis et al. 1996). 6AM hydrolysis in horse blood could be mediated by both AChE and carboxylesterase (CE), the latter of which is present in high amounts in horse plasma (Li et al. 2005). An additional degradation route for 6AM in blood might involve chemical hydrolysis. In a study examining 6AM stability in hair extraction incubation media, the analyte was observed to decline by 6.3% when incubated in pH 7.0 phosphate buffer at 45°C for 18 hours. Hydrolysis increased substantially at pH 8.0, where 6AM had declined by 33% over the same period (Romolo et al. 2003). In unpreserved horse blood stored at 20 and 4°C the pH rose from 7.6 on day 0 to 7.8 by day 7 and thus chemical hydrolysis might be expected to occur.

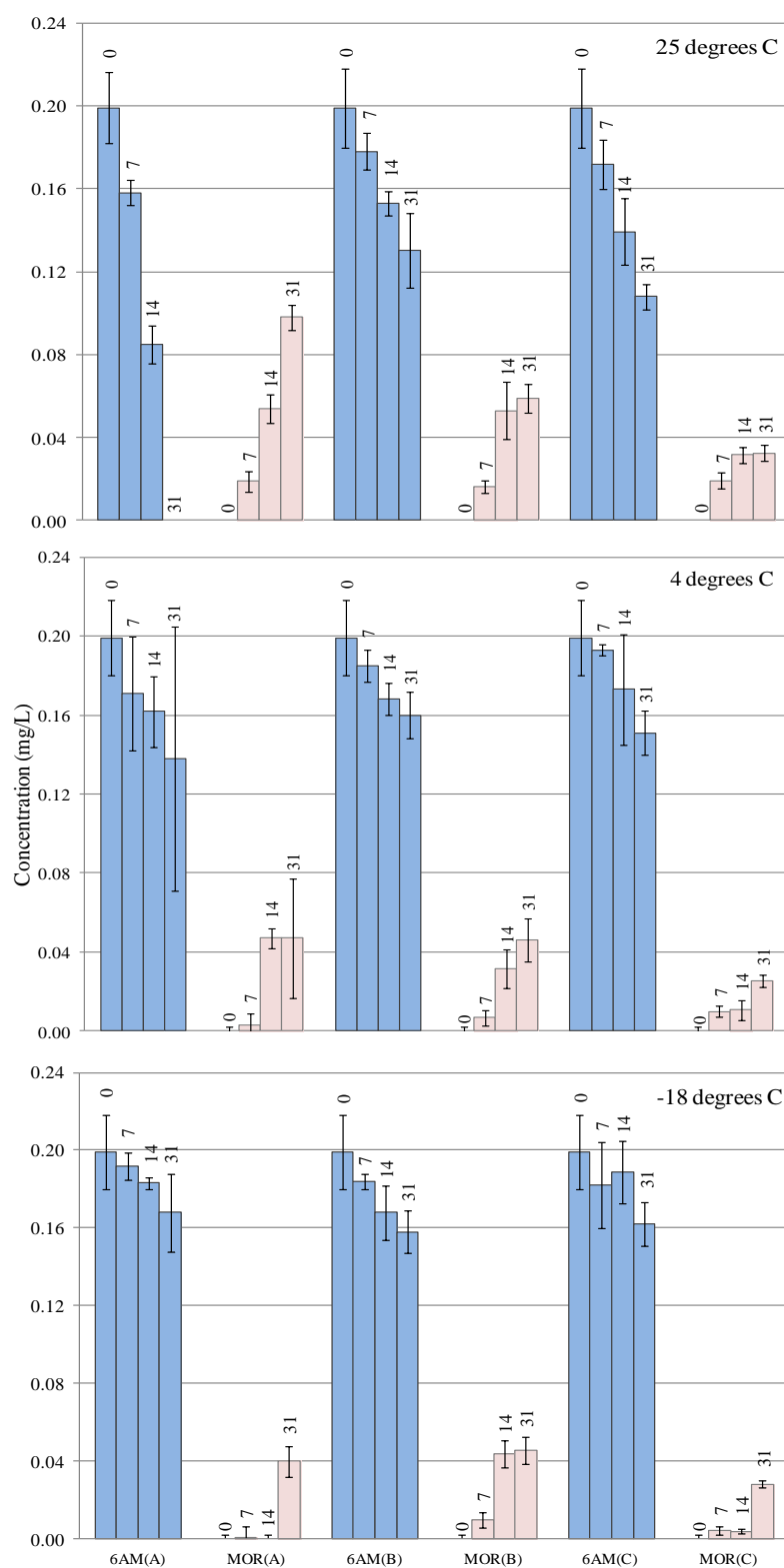


Figure 17 Mean concentrations of 6AM and morphine (MOR) in deer muscle homogenised with (A) water; (B) water+2% NaF, and; (C) buffer+2% NaF stored at 25, 4 and -18°C for 31 days

Error bars = standard deviation (analysis day next to error bar)

There is evidence to suggest that the *in vitro* hydrolysis of 6AM by AChE should not occur in a blank blood sample that has been fortified with 6AM. Salmon et al. (1999) demonstrated that AChE could hydrolyse 6AM only when the metabolite had been produced from heroin within its active site. When 6AM was incubated with erythrocyte AChE as a substrate, no hydrolysis was observed. Whether the same would be true for CE is not known. The results of the present study suggest that enzyme mediated hydrolysis was substantial in horse blood because the addition of preservative increased the stability of 6AM significantly at every temperature. The concentration of NaF further influenced 6AM stability in blood. The rate of degradation observed in the 1% NaF condition was significantly more rapid than in the 2% condition following storage at 20 and 4°C ($p = .05$). Given that the pH of preserved blood was consistently higher than that of unpreserved blood (+ 0.2), chemical hydrolysis would appear to have played a lesser role in the degradation of 6AM compared with enzyme mediated hydrolysis. Further, chemical hydrolysis should be more effectively retarded at frozen temperatures yet the concentration of 6AM had declined by 46% in unpreserved frozen horse blood by the end of the study period. In preserved frozen samples 6AM was stable for the study period. There is very little data on the effect of NaF on 6AM stability in human blood. In a single report the presence of NaF was observed to reduce but not prevent the degradation of 6AM in spiked post-mortem blood samples stored at 4°C (Wathanafa and Cooper 2010). The addition of 1 – 2% NaF has been shown to decrease AChE activity in human blood by only 40 – 50% (Baselt et al. 1985). Given this partial inhibition the continuation of 6AM hydrolysis would be expected to occur in human blood despite preservation.

Rapid degradation of 6AM has been reported in spiked unpreserved human blood. Boy et al. (2008) reported an 80% decline within 7 days following storage at 4°C. In horse blood stored under the same conditions and for the same time period 6AM was observed to decline by 54%. Boy et al. (2008) did not specify the pH of the human blood used in the study. Given the additional hydrolytic pathway provided by horse blood the more rapid decline observed in human blood is somewhat surprising. It also appears to contradict evidence suggesting that AChE does not hydrolyse 6AM unless produced from heroin within its active site (Salmon et al. 1999). Either the observation made by Salmon and co workers does not hold true in blood samples or an alternative mechanism is responsible for the degradation of 6AM. One potential explanation might

relate to the presence of lymphocytes, which have been implicated in the conversion of 6AM to morphine within cell cultures (Hutchinson and Somogyi 2002). The mechanism responsible was found to be non-enzymatic since non-selective esterase inhibitors as well as a selective erythrocyte AChE inhibitor did not reduce the rate of 6AM degradation within cells. Hutchinson and Somogyi (2002) tentatively attributed the observation to reactions or bi-products associated with lymphocyte activity. Future studies on 6AM stability in human blood are required to clarify the degradation mechanisms occurring in this matrix.

The observation that morphine did not account for the total amount of 6AM lost in blood was unexpected. The concentrations generally appeared to be stable following peak or they declined slightly, the latter was observed particularly at 20 and 4°C. This might indicate that morphine was not as stable in this matrix as it has been previously reported to be in fresh human blood (Skopp et al. 2001b). Unfortunately the stability of morphine alone was not examined in the present study.

As with blood, the stability of 6AM in sheep VH was seen to increase with decreasing storage temperature. Irrespective of temperature however, 6AM stability was poor in unpreserved sheep VH (UVH), with losses of 61, 52 and 42% observed by day 7 in samples stored at 20, 4 and -18°C respectively. The addition of NaF to VH (PVH) had a substantial effect on the stability of 6AM at every temperature. In PVH stored at 20°C a loss of only 22% was observed by day 7 and the analyte could still be detected by the end of the study period. At 4°C 6AM was stable for 35 days (6% loss) and moderately stable thereafter. Comparison of regression slopes showed the rate of decline to be significantly slower in PVH compared with UVH at 20 and 4°C ($p = .05$). Even at -18°C the effect of NaF was significant with 5 and 90% of the initial 6AM concentration detected in UVH and PVH respectively at the end of the study period. The effect of fluoride on the long-term stability of 6AM in frozen VH has been indicated previously; in human VH obtained from forensic cases stored with and without the addition of potassium fluoride, 6AM could only be detected in the preserved samples following 12 months storage at -18°C (Holmgren et al. 2004).

The extent of degradation observed in unpreserved sheep VH is in contrast with the results of Pragst et al. (1999), who reported there to be no remarkable degradation of

6AM in spiked human VH stored at 4 and 0°C for 7 days. Although the pH of the human VH used in Pragst's study was not specified, the pH of human VH in 40 cases analysed by Antonides et al. (2007) was reported to be approximately 7.0. The pH measured in unpreserved sheep VH in this study was somewhat higher; in samples stored at 20 and 4°C pH values ranged between 7.0 and 7.8 over the study period. Some spontaneous hydrolysis of 6AM to morphine would be expected to occur within this pH range. However, the addition of NaF to sheep VH actually increased the starting pH of the VH to 7.9. The significant increase in the stability of 6AM observed in preserved samples clearly indicates that pH mediated hydrolysis was not the predominating mechanism of degradation. Given the lack of esterase activity in VH the results might be more indicative of bacterial contamination. Opiate analogues possessing a free phenolic group at the C-3, like morphine and 6AM, have been shown to undergo microbial oxidation (Stabler and Bruce 1998). Fluoride inhibits bacterial growth and would account for the significant increase in the stability of 6AM observed in preserved samples. Aseptic methods were not used during sample collection so bacterial contamination is a possibility. Significant bacterial growth in the VH would not necessarily be expected given that both human and animal VH are known to have inherent antibacterial properties and do not generally support bacterial growth *in vitro* (Davey et al. 1987; Egger et al. 1997). However, a putrid odour was noted with many of the sheep VH samples after 7 days of storage at 20 and 4°C indicating significant putrefaction and suggestive of bacterial growth. Although this study could not confirm the exact mechanism by which NaF increased the stability of 6AM in sheep VH, these results, as well as those of Holmgren (2004) for human VH, clearly demonstrate that its addition is a necessity for the reliable determination of 6AM in VH over time, even when samples are stored at -18°C.

In sheep VH morphine accounted for the total amount of 6AM lost in preserved samples stored at 4 and -18°C. In unpreserved samples and in samples stored at 20°C morphine typically accounted for just over half the amount of 6AM lost. One possible explanation might relate to the formation of an additional product or products resulting from oxidation reactions in the presence of bacteria. The microbial oxidation of morphine has been reported to result in the formation of pseudomorphine (Stabler and Bruce 1998), the same product formed from the chemical oxidation of morphine (Bentley and Dyke

1959). The microbial oxidation product of 6AM was not characterised in the Stabler and Bruce study.

Compared with horse blood and sheep VH, 6AM was found to be more stable in deer muscle stored at 20 and 4°C both with and without preservative. The most significant decline in the concentration of 6AM was observed in unpreserved deer muscle (UM) stored at 25°C where a decline of 21, 57 and 100% was observed on day 7, 14 and 31 respectively. The poor stability observed in these samples might be partly attributed to the pH of the UM, which rose from 5.7 at the start of the study to 8.2 on day 14 and then decreased to pH 7.6 at day 31. Chemical hydrolysis of 6AM would be expected to occur within this pH range, particularly at a pH \geq 8.0 (Romolo et al. 2003). In whole (intact) deer tissue samples (not homogenised) stored at 25°C the pH change was less than that observed in unpreserved homogenates, increasing from 5.7 on day 0 to a maximum of 7.0 on day 31 (Figure 11). Given the slower rate of putrefaction apparent in intact deer tissue, it might be inferred that the stability of 6AM would be increased in intact case material stored under the same conditions.

The pH of UM stored at 4°C also showed a marked change during the study period, rising to pH 6.8 by day 7. In these samples 6AM had declined by 31% by the end of the study period. It is unlikely that the increase in pH observed in UM stored at 4°C would fully account for the observed 6AM decline. Cocaine, which hydrolyses more rapidly than 6AM at equivalent pH values (Romolo et al. 2003) was found to be very stable (loss = 0 - 2%) under the same conditions (Chapter 4). Muscle, like VH, lacks esterase activity and the degradation of 6AM observed at 4°C and 25°C might have been more influenced by bacterial action. As with VH, aseptic methods were not used in the collection of deer tissue samples. This hypothesis is supported by the observation that the addition of NaF to water homogenates (PM) and buffered homogenates (BPM) increased the stability of 6AM substantially at 25°C and 4°C. In PM and BPM the pH changed only marginally (+ 0.2 units) during the study period irrespective of storage temperature, which would further contribute to the increased stability of 6AM in these samples. Surprisingly, the degradation profiles of 6AM in preserved deer muscle (both PM and BPM) stored at 4°C and -18°C were very similar, both demonstrating a steady decline in 6AM over the study period. It is unclear why 6AM continued to degrade in preserved muscle stored at -18°C when in blood and VH the combination of NaF and

frozen storage effectively stabilised the concentration of 6AM (maximum loss = 10%) over a longer period (84 days).

The amount of morphine produced did not account for the total amount of 6AM lost in samples stored at 25°C, particularly without the addition of NaF. As discussed earlier in this chapter this is potentially indicative of the formation of additional products at the higher storage temperature.

5.7 Conclusion

The results obtained in this study highlight the importance of NaF addition to horse blood in the preparation of calibration and check standards, particularly if these standards are prepared in bulk and stored prior to use. The concentration of NaF added to horse blood is also an important consideration and from these results a minimum of 2% is recommended. The combination of frozen storage and NaF addition provided optimal conditions for 6AM stability in horse blood. The addition of NaF to sheep VH was found to be essential for the reliable determination of 6AM over time and in combination with frozen storage provided optimal conditions for 6AM stability in this matrix. The stability of 6AM in muscle tissue homogenates was increased by the addition of NaF. Storage at temperatures of 4°C and below was essential in reducing the rate of 6AM decline in deer muscle tissue.

Chapter 6.0 The distribution of cocaine, benzoylecgonine and cocaethylene in whole thigh muscle (*rectus femoris*), cardiac muscle, vitreous humour and blood in cocaine using decedents

6.1 Abstract

The primary aim of this study was to examine the suitability of skeletal thigh muscle for the determination of opiates and cocaine in drug related deaths. Unfortunately the opportunity to examine the distribution of opiates in muscle did not arise. In nine cases in which urine and/or cardiac blood screened positive for cocaine, the distribution of cocaine, benzoylecgonine (BZE) and cocaethylene (COET) was examined in the *rectus femoris* thigh muscle (ThM), cardiac muscle (CM), femoral blood (FB), cardiac blood (CB) and vitreous humour (VH). In seven of these cases one or more analyte could be detected in ThM (cocaine, $n = 6$; BZE, $n = 7$; COET, $n = 5$). The spatial distribution of the analytes was examined across the whole *rectus femoris*; the muscle was sectioned into 12 roughly equal segments, each of which was analysed following homogenisation. Tissue and bio-fluid samples were extracted by SPE with confirmation and quantification carried out using GC-MS/MS. No significant variation was observed in the concentration of cocaine, BZE or COET throughout the muscle in each of the cases analysed. The results reported here are in contrast to a previous study (Williams and Pounder 1997) in which great variation in the concentration of some basic drugs was observed in thigh muscle. Analyte concentrations in ThM and VH correlated well those in FB ($p < .01$). In general, the concentrations of cocaine and COET followed the order $VH > CM > ThM > FB \geq CB$. Mean cocaine concentrations measured in VH were significantly higher than in FB ($p = .016$) and ThM ($p = .002$). Less inter-matrix variation was observed in the concentrations of BZE and COET. The concentration of BZE exceeded that of cocaine in all matrices and in all cases except in one where death was suspected to have occurred rapidly following drug intake. In this case the cocaine/BZE ratio measured in muscle was substantially higher (2.7) than that measured in FB (0.97). Given that the concentrations of cocaine and its metabolites were uniformly distributed throughout the muscle and considering the good correlation observed between muscle and blood, muscle could be of interpretive value in cocaine related deaths. The use of VH as a quantitative specimen is also discussed.

6.2 Introduction

The selection of appropriate toxicological specimens is vital for the interpretation of post-mortem cocaine concentrations. As a consequence of the competing processes of post-mortem tissue release and continuing chemical and enzymatic breakdown, concentrations of cocaine measured in post-mortem blood do not reflect those at the time of death (Hearn et al. 1991b; McKinney et al. 1995). It is generally agreed that the interpretation of isolated blood cocaine measurements cannot be relied upon (Hearn et al. 1991b; Logan et al. 1997; Karch 2009). Alternative tissues less affected by redistribution and change in the early post-mortem period may provide more reliable specimens in the determination of cocaine and its metabolites. Analysis of skeletal muscle as a post-mortem toxicological specimen has potential advantages, 1) even in burned, putrefied, mummified or exhumed bodies, where body fluids and other conventional toxicological samples are typically missing, muscle tissue is usually available (Christensen et al. 1985); 2) thigh muscle is located away from drug reservoirs in the central organs and so affected by post-mortem change and redistribution later than blood, and; 3) muscle lacks esterase activity so ester containing drugs such as cocaine should have increased stability in this tissue.

The increased *in vitro* stability of cocaine in deer muscle as compared with horse blood was demonstrated in Chapter 4. Similar findings have been reported by Moriya and Hashimoto (1996) in a rabbit model. Despite the potential advantages of skeletal muscle tissue for cocaine determinations, cocaine concentration data in this tissue is extremely limited and often reports are based on only a few cases or on isolated cases (Poklis et al. 1987; Garriott 1991; Moriya and Hashimoto 1996). The lack of interest in muscle tissue as a toxicological specimen may be attributed to the fact that great variation in the concentration of some basic drugs has been observed between different muscles and at different locations within thigh muscle (Williams and Pounder 1997; Langford et al. 1998). Consequently, the use of muscle tissue has been considered only for qualitative drug determination and not for the quantitative corroboration of a blood sample or as a quantitative alternative to blood. In these studies only a limited number of drugs were investigated, predominantly tricyclic antidepressants and benzodiazepines. The within- or between-muscle distribution of most drugs of abuse, including cocaine, has not been reported.

The primary aim of this study was to examine the distribution of cocaine, BZE and COET throughout the *rectus femoris* muscle (mid-anterior quadriceps) in cocaine related deaths. The relationship between the concentrations measured in the muscle and in femoral blood was examined. The study also reports the concentrations measured in vitreous humour, a matrix in which cocaine has been previously shown to have increased stability (Chapter 4; Mackey-Bojack et al. 2000; Duer et al. 2006; Antonides et al. 2006), and in cardiac muscle, a specimen in which toxicological data is extremely limited.

6.3 Methods

6.3.1 Specimen collection

All of the cases analysed in this study (n = 9) were submitted for autopsy at the Medicolegal Institute (IML), Sao Paulo, Brazil, between December 2009 and August 2010. Ethical approval for this study was obtained from Bournemouth University Ethics Committee and from the National Brazilian Ethics Council. Suspected cases of drug poisoning or cocaine involvement were identified prior to autopsy from police reports. Prior to sample collection informed consent was obtained from relatives/next of kin/person with authority over the body. Once the samples were collected, urine, if available, was screened for BZE using the Varian OneCup screen kit⁵ (Varian Inc, Lake Forest, CA) which has a cut-off concentration of 0.3 mg/L. Secondary screening was carried out on cardiac blood using the Randox Analyser (Randox Brazil Ltd., Sao Paulo, Brazil). Blood alcohol analysis was carried out at the Forensic Toxicology Centre of the Forensic Institute of the State of Sao Paulo (Brazil).

The *rectus femoris* muscle was removed in its entirety by cutting at the distal and proximal tendons. The muscle was wrapped in plastic film with the proximal and distal ends clearly marked on the packaging. A 2 cm square segment of cardiac muscle was cut from the mid part of the interventricular septum. Femoral blood and vitreous humour was collected from the left and right side of the body and stored separately for individual analysis. The femoral vein was cross clamped proximally and blood from the severed vessel was collected into a container. Cardiac blood was collected from the

cardiac chambers and pooled. Blood and VH samples were preserved with 2% NaF. All samples were stored at -20°C until analysis.

6.3.2 Chemicals and reagents

Cocaine, BZE, COET, cocaine-d3 and BZE-d8 were purchased from Cerilliant Corporation (Roundrock, Texas). *Isolute* SPE cartridges (10 mL) with 130mg HCL sorbent were generously provided by Biotage (Uppsala, Sweden; Cat# 902-0013-H). Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) + 1% TMCS was purchased from Sigma-Aldrich (Poole, UK). Concentrated hydrochloric acid (37%), ammonia solution (33%) and all other reagents and solvents were of analytical grade obtained from MERCK (Darmstadt, Germany). 1 M phosphate buffer (pH 6.0) was prepared with the addition of 3% sodium fluoride.

6.3.3 Standards and controls

A seven point calibration curve was prepared in the blank matrix containing cocaine, BZE and COET at concentrations ranging from 0.01 to 1.0 mg/L (mg/0.5g tissue). Quality control samples were prepared at 0.03 and 0.4 mg/L (mg/0.5 g) using independently prepared standard solutions and run concurrently with the curve and each batch of sample analyses.

6.3.4 Sample analyses

All samples were extracted at the Faculty of Pharmaceutical Sciences, University of Sao Paulo. Dried extracts were transported to Bournemouth University for GC-MS analysis. Prior to sectioning of thigh muscle, all visible fat and connective tissue was removed from the muscle surface. The muscle was then sectioned into 12 approximately equal segments according to the diagram in Figure 18. One segment in each case was further sub-divided into four segments giving a total of 15 muscle samples. A sectioned muscle is shown in the photograph in Figure19. Each segment was finely chopped with scissors and weighed following removal of any remaining connective tissue. Phosphate buffer (1 M; pH 6.0) containing 2% NaF was added to each segment at a volume three times that of the segment weight. The segments were homogenised using a Turrax Tensao (TE-102) homogeniser (Tecnal Equipamentos Para Laboratoria Ltd, Sao Paulo) at 18,000 rpm for 5 minutes and then at 22,000 rpm until the sample was smooth and homogeneous. Two grams of homogenate (equivalent to 0.5 g tissue) was weighed in

duplicate in 15 mL polypropylene centrifuge tubes and kept on ice until all homogenates had been prepared. With the exception of blank samples, 150 ng of internal standard mix (cocaine-d3/BZE-d8) was added to each 2 g homogenate, to 1 mL of blood and 1mL of VH from real cases and to tissue and blood calibration and QC standards. Analyte extraction was performed by SPE with confirmation and quantification by GC-MS/MS. A full description of the sample preparation procedure and analytical methods is given in Section 3.9.

6.3.5 Statistical analyses

All statistical analyses and calculations were carried out using SPSS v.18. To evaluate analyte distribution throughout ThM the Shapiro-Wilks test was performed on log-transformed data to reduce heteroscedasticity. Non-significant values ($> .05$) confirmed normality. Bivariate correlation analysis was performed on non-log transformed data with Kendall's Tau coefficients (τ) reported for cocaine and COET (non-parametric data groups) and Pearson's correlation coefficients reported for BZE (parametric data group); $p < .05$ was considered significant. Comparison of analyte concentration measured in the different matrices was achieved using the related-samples Wilcoxon signed-rank test. An alpha probability (p) $< .05$ was considered significant for a one pair comparison. Where a family of comparisons was made, the alpha probability was divided by the number of pairs. For the purpose of statistical comparison and correlation analysis, concentrations that were below the limit of quantification (LOQ; defined as the concentration of the lowest calibrator) were included in the data analysis.

6.4 Results

The circumstances surrounding each case reviewed in the current study are reported in Table 24. Of the nine cases, eight were death by trauma (majority by fatal gunfire) and not as a direct result of cocaine use. In case 7 the victim was found to have collapsed in the middle of a road and the post-mortem examination revealed no injury or underlying illness. Death was suspected to be due to drug overdose. Other than THC, cocaine was the only drug detected in case 7. In each case the route of administration was unknown although injection puncture marks were not visible in any case. In three cases (5, 6 and 8) a femoral blood sample was not available for analysis.

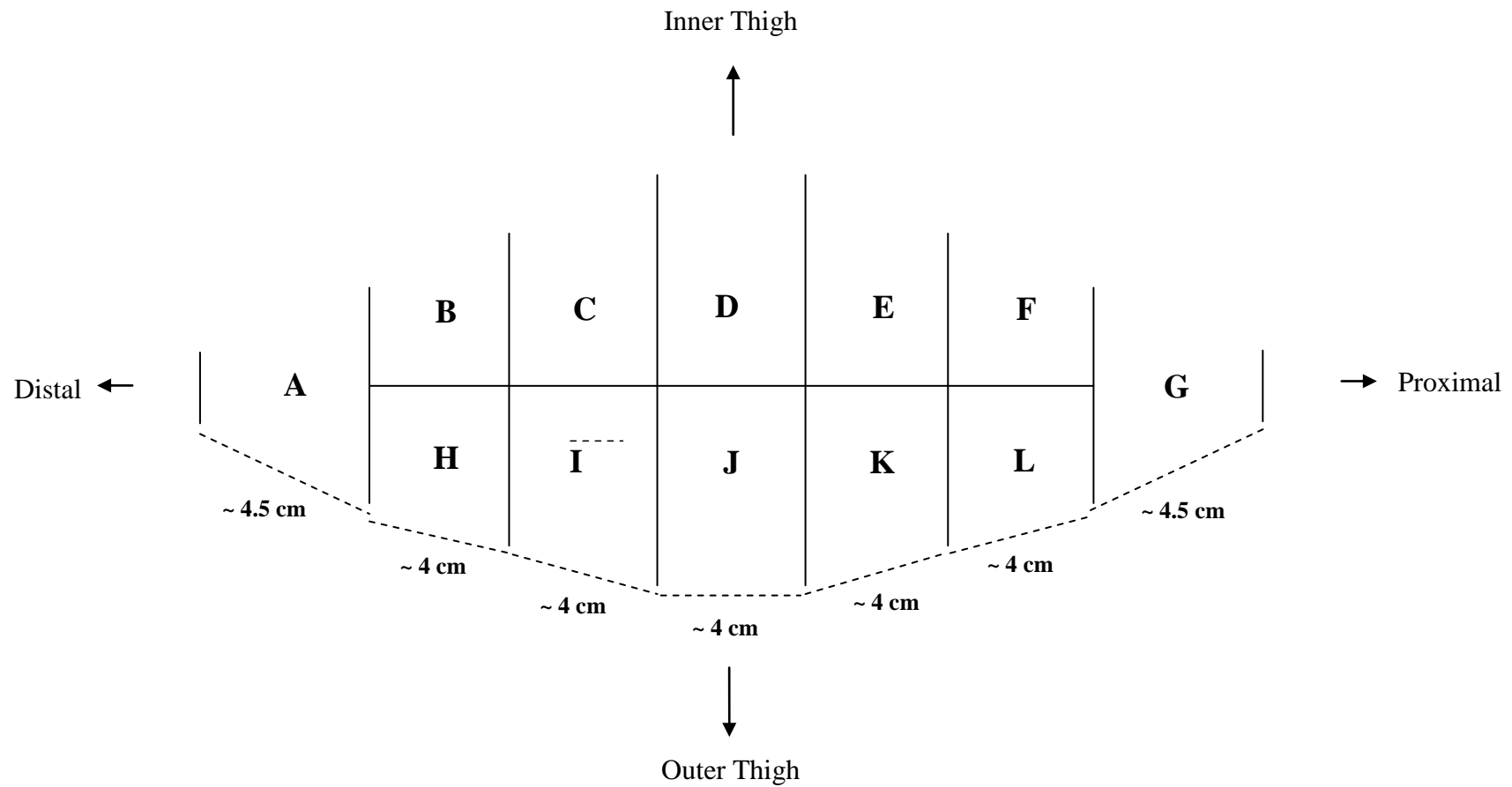


Figure 18 *Rectus femoris* sectioning diagram

Note: One section was further sub-divided into four approximately equal sized sections in each case, as shown in Figure 19



Figure 19 Sectioned *rectus femoris* muscle

Note: segment D subdivided

In seven of the nine cases analysed, one or more of the analytes of interest was detected in the *rectus femoris*. BZE was detected in the muscle in each of these seven cases at a concentration above the LOQ. Cocaine was detected in six cases although in case 4 it was only detected in five of the 15 segments analysed. COET was detected in muscle in four cases. The distribution of each analyte throughout the muscle was assessed using the percentage relative standard deviation (%RSD) calculated from analyte concentration and by determination of the normality of the distribution using the Shapiro-Wilks test on log-transformed data. A $RSD \leq 15\%$ is a common limit of acceptability to ensure precision between replicate toxicological measurements. Shapiro-Wilks significance values $> .05$ (i.e. not significant) provided confirmation that the spatial distribution was normal, thus demonstrating that the concentrations did not vary significantly throughout the muscle bulk. Mean concentration and statistical data are presented in Table 25. Owing to the low number of positive ThM samples in case 4, the normality of the distribution was not examined. The mean concentration data and %RSD over the five cocaine positive segments is reported. The concentrations measured in the 12 main muscle segments in each case are tabulated in Appendix 28. The concentrations measured in subsectioned muscle segments are tabulated in Appendix 29.

In a few cases a single result relating to a single muscle segment was removed for normality testing. In case 1 BZE ranged from 0.82 – 1.1 mg/kg with a mean of 0.88 mg/kg. In removing the highest result the range was 0.82 – 0.90 mg/kg with a mean of 0.87 mg/kg. In case 3 the removal of the highest BZE result, 1.7 mg/kg, gave a range of 1.3 – 1.4 mg/kg. A single outlier was also removed in case 6 for COET (0.041 mg/kg) giving a range of 0.013 – 0.022 mg/kg. All removed data points were included in the mean calculation and in determination of the %RSD (Table 25). Analyte distribution in each of the seven cases was determined to be normal (Shapiro-Wilks significance values $> .05$) with the exception of BZE in case 1 where a significant Shapiro-Wilks result was obtained (.035). In this case, a very low RSD (8%) was obtained even with the inclusion of the outlying result (1.1 mg/kg) demonstrating that the concentration did not in fact show significant variation throughout the muscle bulk. Taking the two extremes of the BZE range (0.82 and 1.1 mg/kg) in case 1, the deviation from the mean was well within the 15 – 20% limit considered acceptable for replicate analytical measurements (Cooper et al. 2010).

Table 24 Circumstances surrounding death in nine cocaine involved deaths

Case	Sex	Age	Wt (kg)	Ht. (m)	BMI	Race	Case circumstances	PMI	Other drugs
1	M	21	70	1.72	23.7	W	GSW – robbery, gunfire by police, died at scene	~ 8h	BAC (1.2 g/L), THC
2	M	?	75	1.75	24.5	AB	GSW – robbery, gunfire by police, died at scene	~ 10h	BAC (1.6 g/L), THC
3	M	34	70	1.71	24	AB	GSW/poly-trauma – homicide, died at scene	≥ 12h	None
4	M	24	72	1.75	23.5	AB	GSW/head trauma – homicide, died at scene	≥ 12h	THC
5	M	32	70	1.7	24.2	AB	GSW/head trauma – resisting arrest, gunfire by police, died at scene	~ 12h	None
6	M	36	75	1.7	26	W	GSW/stomach trauma – homicide died in hospital	15-20h	None
7	M	22	73	1.73	24.4	AB	Unknown – victim found dead in middle of road, no visible trauma or underlying illness, suspected overdose	10-12h	THC
8	M	22	75	1.7	26	AB	GSW/head trauma – homicide, died at scene	10-12h	THC
9	M	15	63	1.7	21.8	W	Head trauma – hit by car, died on impact	~ 10h	THC

PMI: Post-mortem interval; Race: W – White; AB – Afro Brazilian; GSW: Fatal gunshot wound; BAC: Blood alcohol concentration

There were four instances where the RSD obtained was $> 15\%$; cocaine and COET in case 1, cocaine in case 4 and COET in case 6. In each case the mean concentrations were low (0.013 – 0.050 mg/kg). When the mean value is close to zero, the RSD is sensitive to small variations thus limiting its ability to provide a true indication of variation within a sample set. Normality was demonstrated in each of these instances indicating that there was no significant variation in concentration between the different segments. These findings are significant as they diverge from those of Williams and Pounder (1997), who observed great variation in the concentration of some other basic drugs throughout the thigh muscle.

Analyte concentrations measured in each matrix are displayed graphically in Figure 20 (cocaine), 21 (BZE) and 22 (COET). This data is tabulated in Appendix 30. The results reported for FB and VH represent the mean of the results obtained in left and right samples. Individual left and right results and the percentage deviation from the mean of the two concentrations are tabulated in Appendix 31. In the majority of cases the deviation did not exceed 20%. In a single case the concentration of cocaine in right and left VH differed more substantially with concentrations of 0.71 and 0.35 mg/L measured respectively (deviation 34%). In this case the concentration of BZE was also higher in the right VH sample (1.0 mg/L) compared with the left (0.65 mg/L) although the percentage deviation was less than for cocaine (21%). Whether this finding reflects a real difference in the distribution of these analytes in this case or whether it is the result of an extraction/quantification error is unclear. Results that were close to the LOQ tended to show more variation than at higher concentration, which is to be expected given that variation is amplified at low concentration. With the exception of the VH in case 3, right and left sample results were comparable.

In general, the concentration of cocaine followed the order $VH > CM > ThM > FB \geq CB$, although in one case CM cocaine greatly exceeded the VH cocaine. There appeared to be less variation between matrices in the concentrations of BZE and COET. The mean and median matrix concentration, standard deviation and range for each analyte are displayed in Table 26. With the exception of VH, the median concentration was much lower than the mean both for cocaine and COET, reflecting the positive skew of these data sets.

Table 25 Concentration and within-muscle distribution of cocaine, BZE and COET in *rectus femoris* muscles (n = 7)

Case no.		Concentrations in <i>rectus femoris</i> (ThM)				Shapiro-Wilks		
(no of segments)	Compound	Mean (mg/kg)	Range (mg/kg)	S.D	RSD (%)	Statistic	df	Sig.
1 (n=14)	Cocaine ^A	0.029	0.021 – 0.048	0.009	31	0.913	9	.336
	BZE ^B	0.88	0.82 – 1.1	0.067	8	0.857	13	.035
	COET	0.032	0.021 – 0.050	0.007	22	0.967	14	.831
2 (n=12)	Cocaine	0.046	0.035 – 0.053	0.005	11	0.904	12	.177
	BZE	0.39	0.35 – 0.47	0.032	8	0.934	12	.428
	COET	0.097	0.077 – 0.11	0.010	10	0.919	12	.279
3 (n=15)	Cocaine	0.35	0.32 – 0.40	0.022	6	0.920	15	.193
	BZE ^C	1.4	1.3 – 1.7	0.11	8	0.903	14	.125
	COET	<0.01						
4 (n=15)	Cocaine (n=5)	0.018	0.014 – 0.024	0.005	28			
	BZE	0.098	0.075 – 0.11	0.010	10	0.944	15	.430
	COET	0						
5 (n=15)	Cocaine	0						
	BZE	0.29	0.26 – 0.32	0.015	5	0.950	15	.525
	COET	0						
6 (n=15)	Cocaine	0.27	0.21 – 0.33	0.032	12	0.984	15	.989
	BZE	0.84	0.72 – 0.91	0.052	6	0.929	15	.262
	COET ^D	0.018	0.013 – 0.041	0.007	39	0.903	14	.125
7 (n=14)	Cocaine	0.38	0.32 – 0.47	0.040	10	0.980	14	.972
	BZE	0.14	0.13 – 0.16	0.008	6	0.947	14	.518
	COET	<0.01						

Shapiro-Wilks sig. values > .05 = normal distribution

^A Only 9 results for cocaine due to technical difficulty; ^B Single result (1.1 mg/kg) removed prior to normality (range = 0.82 – 0.9 mg/kg);^C Single result (1.7 mg/kg) removed prior to normality testing (range = 1.3 – 1.4 mg/kg); ^D Single result (0.041 mg/kg) removed prior to normality testing (range = 0.013 – 0.022 mg/kg)

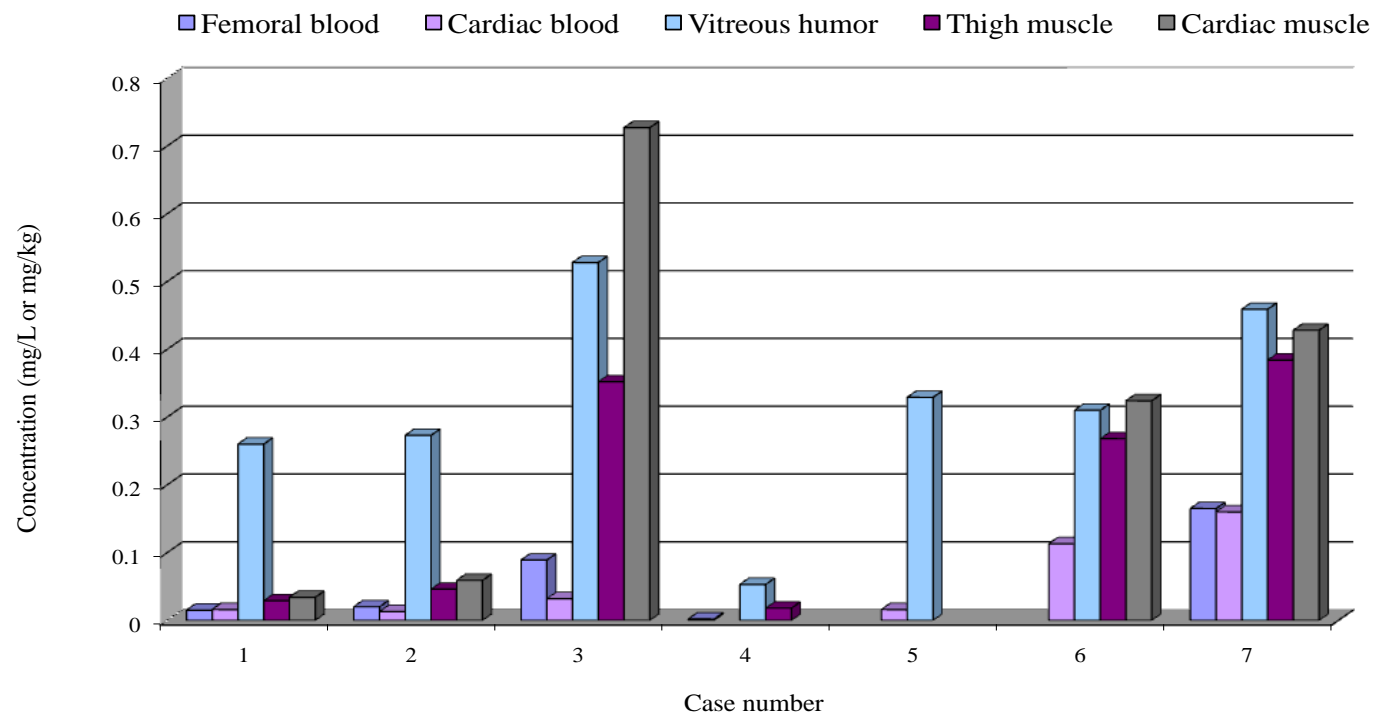


Figure 20 Tissue distribution of cocaine in seven cases of cocaine involved death

FB and VH concentration represents the mean of left and right sample concentration; mean of duplicate analyses reported for all matrices

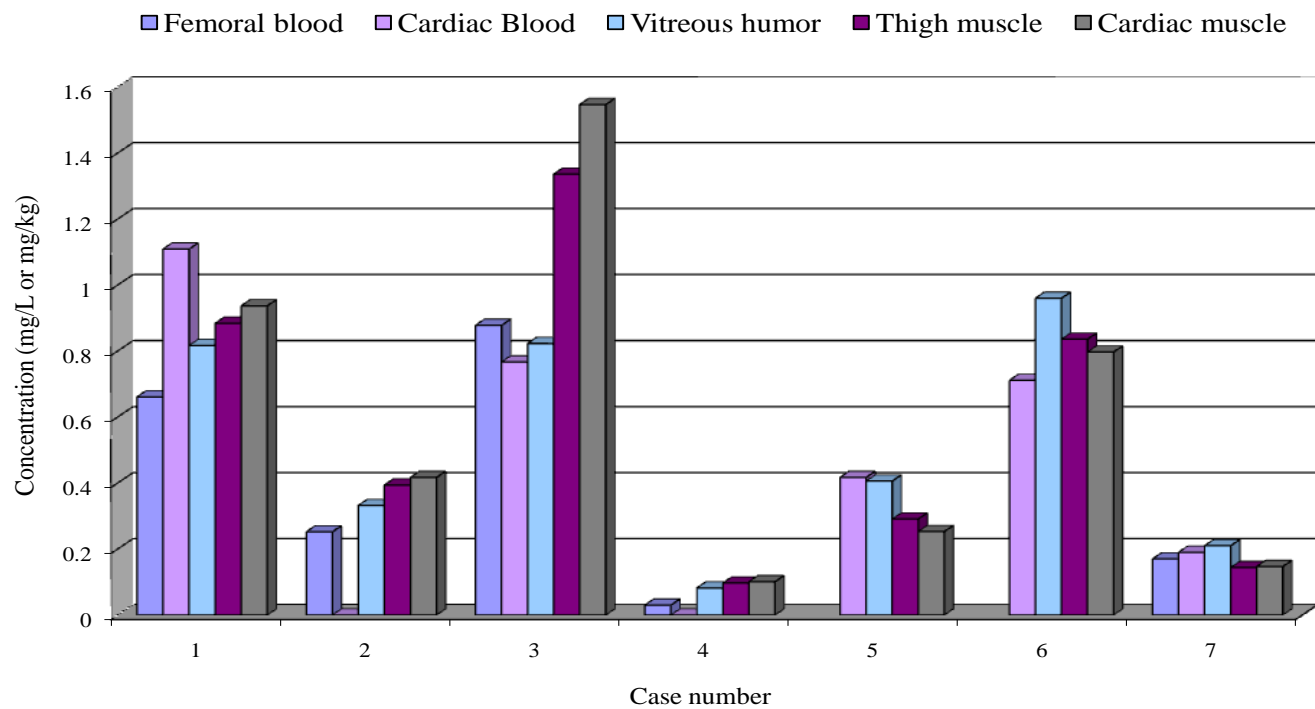


Figure 21 Tissue distribution of BZE in seven cases of cocaine involved death

FB and VH concentration represents the mean of left and right sample concentration; mean of duplicate analyses reported for all matrices

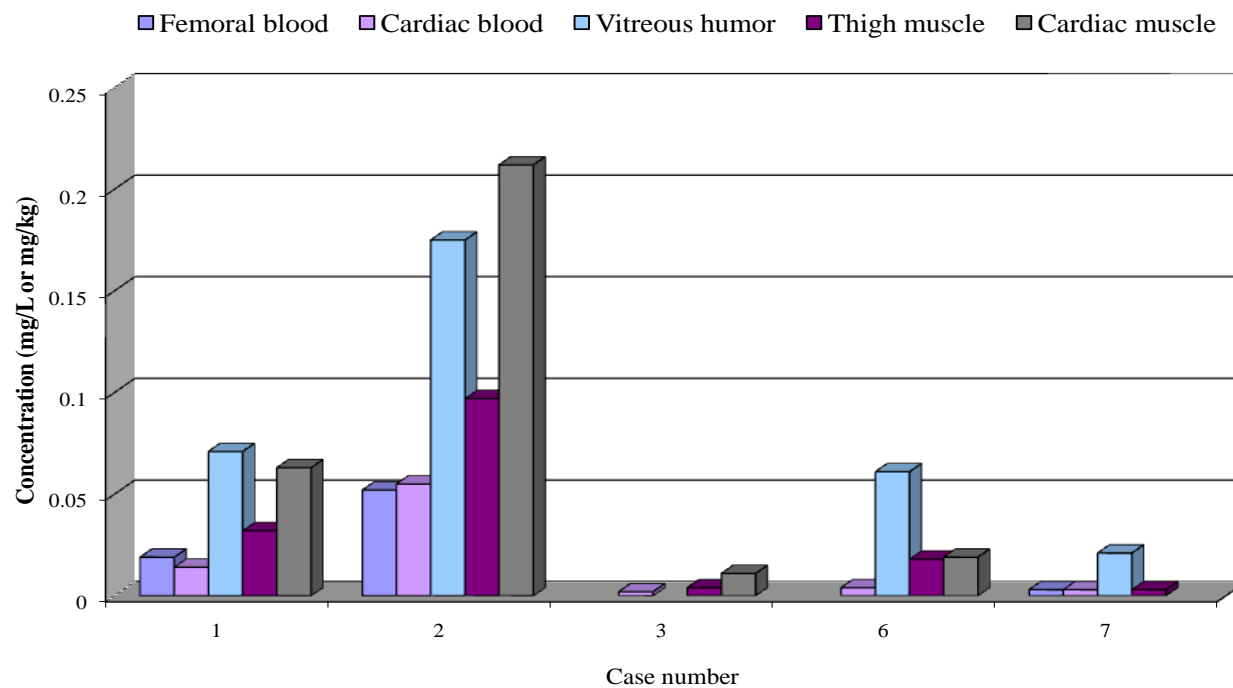


Figure 22 Tissue distribution of COET in five cases of cocaine involved death

FB and VH concentration represents the mean of left and right sample concentration; mean of duplicate analyses reported for all matrices

For cocaine there appeared to be large differences between the mean concentrations measured in blood, muscle and VH. This difference was more marked when considering the median (mdn) concentration. To assess the significance of these differences the related-samples Wilcoxon signed-rank test was applied to three matrix pairs; FB-VH, FB-ThM and ThM-VH. The test was not performed on all matrix pairs owing to the potential increasing risk of Type 1 error. Repeated pairs comparison reduces the alpha probability ($p < .05$ significance for one pair) by a factor of one for each additional pair. The probability (p) for the three comparisons made here was thus reduced by a factor of three resulting in $p < .02$ being significant. Differences between the cocaine concentration in FB (mean: 0.049 mg/L; mdn: 0.018 mg/L) and ThM (mean: 0.12 mg/kg; mdn: 0.030 mg/kg) were not significant ($p = .031$), whereas the concentration in VH (mean: 0.25 mg/L; mdn: 0.27 mg/kg) differed significantly from FB ($p = .016$) and ThM ($p = .002$). As would be expected from the concentration data, the mean VH to blood ratios for cocaine were high (VH/FB: 13 (2.8 – 27); VH/CB: 12 (1.4 – 21); Table 27). The muscle to blood ratios were also high with means ranging between 3.2 and 5.0 (overall range: 0 – 23). The median ratios were not remarkably different from the mean (Table 27). Mean matrix ratios for BZE were closer to unity (mean of all matrix ratios: 1.0) and demonstrated a much narrower range (0.7 – 1.8) compared with cocaine. For COET the mean muscle to blood ratios (1.5 – 3.1) were marginally lower compared with the VH to blood ratios (VH/FB: 4.7; VH/CB: 5.2; Table 27). In determining the correlation of the analytes in the different matrices, analytes that were detected at a level below the LOQ were included. Table 28 gives the correlation coefficients and corresponding significance levels for each of the analytes in all paired matrices. Good correlations were observed between all matrices for cocaine ($p < .05$) and BZE ($p < .01$). With the exception of CM/FB, CM/CB and ThM/CB ($p > .05$) correlations were also observed for COET ($p < .05$).

With the exception of case 7, the BZE concentration exceeded the cocaine concentration in every matrix. In case 7, cocaine exceeded BZE in VH, ThM and CM. Owing to the substantial difference between the cocaine/BZE ratios in case 7 compared with all other cases, calculation of the mean ratio in each matrix was carried out with and without the inclusion of this case (Table 29). When excluding case 7, the mean cocaine/BZE ratio in blood and muscle were similar (ThM: 0.14; mdn: 0.10 (range: 0 – 0.32); CM: 0.18; mdn: 0.09 (0 – 0.47); FB: 0.07; mdn: 0.07; (0.02 – 0.1); CB: 0.12; mdn: 0.04 (0 –

0.53)). In VH the mean ratio was higher (0.52; mdn: 0.50 (0.24 – 0.82)). The related-samples Wilcoxon signed-rank test was used to compare the ratio in VH with that measured in FB and ThM ($p < .025$ considered significant for a two pair comparison). The cocaine/BZE ratio in VH was significantly higher than for FB ($p = .01$) and ThM ($p = .015$). When including case 7 in the cocaine/BZE calculation a significant difference was observed between FB (mean: 0.25; mdn: 0.08 (0.02 – 0.97)) and VH (mean: 0.71; mdn: 0.64 (0.24 – 2.2); $p = .012$) but not between ThM (mean: 0.50; mdn: 0.11 (0 – 2.7)) and VH ($p = .109$).

Table 26 Summary concentration data (mg/L; mg/kg) for cocaine, BZE and COET in all matrices

		CB	FB*	VH	TM	CM
Cocaine n=9 cases	Mean	0.040	0.049	0.25	0.12	0.18
	Mdn	0.016	0.018	0.27	0.030	0.034
	SD	0.057	0.066	0.19	0.16	0.26
	Range	0-0.16	0-0.17	0.004-0.53	0-0.38	0-0.73
BZE n=9 cases	Mean	0.39	0.33	0.41	0.44	0.47
	Mdn	0.22	0.21	0.33	0.29	0.25
	SD	0.39	0.36	0.37	0.47	0.53
	Range	0-1.1	0-0.88	0.011-0.96	0-1.34	0-1.55
COET n=6 cases	Mean	0.014	0.015	0.055	0.026	0.051
	Mdn	0.006	0.003	0.041	0.011	0.015
	SD	0.020	0.022	0.066	0.037	0.082
	Range	0.002-0.055	0-0.052	0-0.18	0-0.097	0-0.21

CB: cardiac blood; FB: femoral blood; VH: vitreous humour; ThM: thigh muscle; CM: cardiac muscle

Mdn: median; SD: standard deviation

*FB sample unavailable in 3 cases (mean of 6 cases reported)

Table 27 Matrix ratios for all analytes by individual case (n=9) with mean, median (mdn), standard deviation (SD) and range for each cases

Matrices	Case no.	1	2	3	4	5	6	7	8	9	Mean	Mdn	SD	Range
ThM/FB	Cocaine	2.0	2.3	4.0	4.0			2.3			2.9	2.3	0.99	2.0 – 4.0
	BZE	1.3	1.6	1.5	3.2			0.85			1.7	1.5	0.90	0.85 – 3.2
	COET	1.7	1.9					1.0			1.5	1.7	0.47	1.0 – 1.9
ThM/CB	Cocaine	1.9	3.5	11			2.4	2.4		0	3.5	2.4	3.8	1.9 – 11
	BZE	0.80	1.8	1.7	1.2	0.70	1.2	0.76		0	1.0	0.98	0.6	0 – 1.7
	COET	2.4	1.8	2.5			4.3	1.0		0	2.0	2.1	1.5	0 – 4.3
ThM/VH	Cocaine	0.12	0.16	0.67	0.15	0	0.87	0.83	0	0	0.31	0.15	0.37	0 – 0.87
	BZE	1.1	1.2	1.6	1.2	0.71	0.86	0.69	0	0	0.82	0.86	0.54	0 – 1.6
	COET	0.46	0.55				0.28	0.14		0	0.29	0.28	0.23	0 – 0.55
ThM/CM	Cocaine	0.88	0.76	0.48			0.83	0.89			0.77	0.83	0.17	0.48 – 0.89
	BZE	0.95	0.94	0.86	0.96	1.2	1.0	0.98			0.98	0.96	0.60	0.86 – 1.2
	COET	0.52	0.46	0.45			0.89				0.58	0.49	0.21	0.45 – 0.89
CM/FB	Cocaine	2.3	3.0	8.2	0			2.6			3.2	2.6	3.0	0 – 8.2
	BZE	1.4	1.7	1.8	3.4			0.86			1.8	1.7	0.94	0.86 – 3.4
	COET	3.3	4.1					0			2.5	3.3	2.2	0 – 4.1
CM/CB	Cocaine	2.1	4.5	23		0	2.9	2.7		0	5.0	2.7	8.0	0 – 23
	BZE	0.84	1.9	2.0		0.61	1.1	0.77		0	1.0	0.84	0.72	0 – 2.0
	COET	4.5	3.9	5.5			4.8	0		0	3.1	4.2	2.5	0 – 5.5
CM/VH	Cocaine	0.13	0.22	1.4	0	0	1.1	0.93	0	0	0.41	0.13	0.55	1 – 1.4
	BZE	1.2	1.3	1.9	1.2	0.62	0.83	0.70	0	0	0.85	0.83	0.61	0 – 1.9
	COET	0.89	1.2				0.31			0	0.60	0.60	0.55	0 – 1.2
FB/CB	Cocaine	0.94	1.5	2.8				1.0		0	1.3	1.0	1.0	0 – 2.8
	BZE	0.60	1.2	1.1	0.38			0.89		0	0.70	0.75	0.46	0 – 1.2
	COET	1.4	0.95	0				1.0		0	0.66	0.95	0.62	0 – 1.4
VH/FB	Cocaine	17	14	5.9	27			2.8			13	14	9.4	2.8 – 27
	BZE	1.2	1.3	0.94	2.7			1.2			1.5	1.2	0.71	0.94 – 2.7
	COET	3.7	3.4					7.0			4.7	3.7	2.0	3.4 – 7.0
VH/CB	Cocaine	16	21	17		21	2.7	2.9		1.4	12	16	8.9	1.4 – 21
	BZE	0.74	1.5	1.1	1.1	0.97	1.4	1.1		3.2	1.4	1.1	0.77	0.74 – 3.2
	COET	5.1	3.2	0			15	7.0		0.50	5.2	4.1	5.6	0 – 15

Table 28 Matrix correlations and significance levels for cocaine, BZE and COET

		ThM/FB	ThM/CB	ThM/VH	ThM/CM	CM/FB	CM/CB	CM/VH	FB/CB	FB/VH	CB/VH
Cocaine	τ	.940	.627	.667	.890	.828	.658	.730	.733	.867	.686
	Sig.(<i>p</i>)	.004*	.012*	.007*	.001*	.011*	.010*	.004*	.019*	.007*	.006*
	n	6	9	9	9	6	9	9	6	6	9
BZE	<i>r</i>	0.992	0.877	0.922	0.995	0.986	0.844	0.880	0.899	0.982	0.931
	Sig. (<i>p</i>)	.000*	.001*	.000*	.000*	.000*	.002*	.001*	.007*	.000*	.000*
	n	6	9	9	9	6	9	9	6	6	9
COET	τ	.738	.467	.733	.966	.667	.552	.690	.738	.949	.733
	Sig. (<i>p</i>)	.038*	.094	.019*	.004*	.059	.063	.028*	.038*	.011*	.019*
	n	5	6	6	6	5	6	6	5	5	6

CB: cardiac blood; FB: femoral blood; VH: vitreous humour; ThM: thigh muscle; CM: cardiac muscle

τ : Kendall's Tau correlation coefficient (non-parametric data group)

r: Pearson's correlation coefficient (parametric data group)

Sig.: point significance for a one-tailed test

*significant *p* value (*p* < .05)

n: number of samples

Table 29 Cocaine to BZE ratios in each matrix with mean, median (mdn), standard deviation (SD) and range given with the inclusion and exclusion of case 7

Case no.	CB	FB	VH	ThM	CM
1	0.01	0.02	0.32	0.03	0.04
2	0.06	0.08	0.82	0.11	0.14
3	0.04	0.10	0.64	0.26	0.47
4	0	0.07	0.65	0.19	0
5	0.04		0.81	0	0
6	0.16		0.32	0.32	0.41
7	0.84	0.97	2.2	2.7	2.9
8			0.36		
9	0.53		0.24		
Mean	0.21	0.25	0.71	0.50	0.57
Mdn	0.02	0.08	0.64	0.11	0.14
SD	0.31	0.40	0.60	0.95	1.1
Range	0-0.84	0.02-0.97	0.24-2.2	0-2.7	0-2.9
Mean (exc. Case 7)	0.12	0.07	0.52	0.14	0.18
Mdn	0.04	0.07	0.50	0.10	0.09
SD (exc. Case 7)	0.19	0.03	0.24	0.13	0.21
Range (exc. Case 7)	0-0.53	0.02-0.1	0.24-0.82	0-0.32	0-0.47

CB: cardiac blood; FB: femoral blood; VH: vitreous humour; ThM: thigh muscle; CM: cardiac muscle

6.5 Discussion

Although muscle is remote from the central body compartment and therefore less likely to show elevated drug concentrations attributable to post-mortem change and redistribution, very few reports have examined drug concentrations in this tissue. In order for muscle to provide a useful quantitative toxicological specimen, drug concentrations measured throughout the muscle bulk should show minimal variation and the concentration should correlate with that in femoral blood. At present little is known about the relationship between blood and muscle drug concentrations in humans and concentration data for cocaine and its metabolites in muscle tissue is extremely limited. To date only a single report has dealt with the distribution of a limited number of basic drugs throughout the

muscle bulk (Williams and Pounder 1997). The muscle distribution of most illicit drugs, including cocaine, has not been reported.

In seven of the cases analysed in the present study (case 1 – 7) one or more of the analytes of interest was detected throughout the muscle bulk. In each case the concentrations did not vary significantly. On a few occasions the result relating to a single muscle segment (not more than one segment result per muscle) was removed to achieve a normal distribution. However, the %RSD in each case was generally low even with the inclusion of the outlier. The largest %RSDs were observed in cases in which the analyte concentration was very low. When the mean value is close to zero, the RSD is sensitive to small variations thus limiting its usefulness. The normality of the distribution and the range, in terms of the interpretive outcome at the extremes of the range, were more important indicators of the variation in these cases. In a real case situation the interpretation of the lowest and highest analytical result in each of the cases studied would not have been different.

The results of the present study diverge from the findings of Williams and Pounder (1997) where significant variation in the concentration of some other basic drugs, mainly tricyclic antidepressants and benzodiazepines, was observed throughout the thigh muscle bulk (specific sampling locations not specified). These authors examined drug distribution in eight overdose cases and reported %RSDs ranging from 10.5% for carbamazepine (range: 10.3 – 17.0 mg/kg) to 50% for thioridazine (0.3 – 2.9 mg/kg). Although the %RSD for carbamazepine is < 20%, the concentration range is wide demonstrating that the variation in concentration throughout the muscle bulk is quite substantial. In six of the seven muscle positive cases analysed in the present study cocaine was an incidental finding with death being due to trauma and not fatal overdose as in the cases examined by Williams and Pounder (1997). The uniform distribution observed in the present study could, in part, be attributed to a delay between drug administration and death with cocaine having sufficient time to distribute into the tissues. Although the exact time between cocaine administration and death is not known for the present cases, the circumstances in some of the cases would imply a delay. In three of the cases the victims were shot by police whilst committing an offence and in another shooting case the victim was not killed instantly but died later in hospital. In case 7, however, death was suspected to be an acute event following drug

intake, although whether death was as a direct result of cocaine intake cannot be confirmed. Given that in this case cocaine was also uniformly distributed throughout the muscle, the time between drug administration and death may not be the most important factor. It may be that the distribution of the analytes in muscle is more heavily influenced by the pattern of previous administration.

Prolonged urinary excretion of both cocaine and BZE has been observed in abstinent chronic cocaine users suggesting that cocaine is accumulated and stored in deep body compartments with slow release back into circulation (Cone and Weddington 1989; Burke et al. 1990). In two cases involving the chronic therapeutic use of temazepam, Langford and Pounder (1998) reported comparable concentrations in several peripheral muscles possibly indicative of tissue equilibration in chronic users. However, in a single case involving the chronic use of fluoxetine, the concentration range was large (0.03 – 0.6 mg/kg, %RSD = 56). The differences observed in the distribution of these two drugs might be attributed to their volumes of distribution (V_d). The V_d of temazepam ($V_d = 0.8 - 1.0$ L/kg) being much lower than that of fluoxetine ($V_d = 20 - 42$ L/kg). The authors found that in general the lower the V_d of an analyte the lower the variation in drug concentration. Williams and Pounder (1997) noted the same trend for within muscle distribution. Compared with fluoxetine the V_d of cocaine (1.9 – 2.4 L/kg) and COET (2.7 – 2.9 L/kg) (Hart et al. 2000) is relatively low which might go some way to explaining the uniform distribution observed here.

In general where the analytes were detected in the FB they were also detected in the muscle (ThM and CM) and correlations were observed between FB and ThM for cocaine ($\tau = .940$; $p < .005$), BZE ($r = .992$; $p < .001$) and COET ($\tau = .738$; $p < .05$). In the cases discussed here, differences observed between the concentrations of cocaine in central and peripheral blood and in central and peripheral muscle were not great. The ThM/CM and FB/CB ratios were 0.77 (0.48 – 0.89) and 1.3 (0 – 2.8) respectively. In each case the post-mortem interval (PMI) was relatively short with death having occurred on average 12 hours (maximum 20 hours) prior to sample collection, remarkably quick compared to the UK where an autopsy may not be carried out for several days following receipt of the body. Thus, the effects of PMR might not be expected to be significant in the present cases, although this cannot be

confirmed. The mean concentration of cocaine in CM (0.18 mg/kg) was much higher than in CB (0.040 mg/L) indicating that cocaine is accumulated and concentrated in this tissue. The difference between cocaine concentrations measured in CM and ThM (0.12 mg/kg) was not remarkable. In a single case report, Poklis et al. (1987) also reported the concentration of myocardial cocaine to be similar to that measured in skeletal muscle. In the present study good correlations were observed for cocaine between CM and ThM ($\tau = .890$, $p < .005$) and between CM and FB ($\tau = .828$, $p < .05$).

In the limited number of reports on cocaine concentrations in muscle and blood, the concentrations measured in muscle are often higher than, or comparable to, those measured in blood (Christensen et al. 1985; Poklis et al. 1987; Garriott 1991). The thigh muscle and blood concentration data reported by Poklis et al. (1987) and Garriott (1991) are summarised in Table 30 alongside the results obtained in thigh muscle and femoral blood in the present study. In all of the cases where cocaine was detected in the muscle in the present study, the concentration was higher than that measured in the corresponding blood sample. The mean ThM/FB ratio was 2.9 (range: 2.0 – 4.0). This finding was in agreement with those of Poklis et al. (1987) who reported thigh muscle to blood (site of collection not stated) ratios of 1.5 – 3.4 in three cases of cocaine overdose (Table 30). In eight cases analysed by Garriott (1991) the mean thigh muscle (not otherwise specified) to aortic blood ratio for cocaine was 1.2 with a range of 0 – 6.5 (Table 30). In two cases of acute cocaine overdose these authors reported very low muscle/blood ratios for cocaine (0.1), and concluded this to be consistent with rapid death and insufficient time for tissue equilibration. In contrast, a relatively high ThM/FB ratio (2.3) was obtained in the present study for the single case in which death was suspected to be an acute event following drug intake (case 7). A high cocaine concentration in the muscle compared with the blood might be attributed to repeated dosing (bingeing) and subsequent tissue accumulation of the drug prior to death. The mean ThM/FB for COET was 1.5 with a narrower range (1.0 – 1.9) compared to cocaine. The mean ThM/FB for BZE was 1.7 (range: 0.85 – 3.2). The mean concentrations of BZE and COET measured in all matrices were not remarkably different from one another and correlations were observed between all matrices for BZE and between most matrices for COET (Table 28). In contrast to the findings of the present

study, Garriott (1991) noted much lower ratios for BZE (mean: 0.26; range: 0 – 0.7; Table 30).

The extent to which BZE distributes into muscle tissues is not known from the literature but owing to its low apparent V_d (0.7 L/kg) (Ambre et al. 1991) and low lipophilicity (BZE is highly ionised at physiological pH ranges) it should not readily distribute from the systemic circulation into surrounding tissues. In three cases analysed by Garriott (1991) BZE was not detected in the muscle yet the corresponding cocaine concentrations were 0.06, 0.15 and 1.06 mg/kg, indicating that if BZE does distribute into the muscle, it does so at a substantially slower rate than cocaine. Skeletal muscle does not contain carboxylesterases that metabolise cocaine to EME (hCE-2) or BZE (hCE-1) (Satoh et al. 2002) so enzymatic breakdown would not be expected to occur in this matrix. The *in vivo* pH of human skeletal muscle is pH 7.0 (Pan et al. 1988; Yoshioka et al. 2002) and thus chemical hydrolysis would be expected to occur in this tissue. The absence of esterase activity combined with the more limited distribution of BZE into the muscle might imply that the majority of the BZE detected in muscle is a result of *in situ* chemical hydrolysis. Thus, the uniform distribution of BZE observed throughout the muscle can likely be attributed to the uniform distribution of cocaine. High BZE concentrations measured in muscle tissue might reflect repeated cocaine intake over a period of hours or days resulting in accumulation of cocaine and subsequent chemical breakdown. Although EME was not analysed in the present study it has been detected at relatively low concentration in muscle tissue (Garriott 1991). Owing to the lack of esterase activity its presence in muscle more likely reflects its distribution from the blood. Although the V_d of EME has not been measured in humans, its V_d in the rat (3.1 L/kg) was lower than that of cocaine (5.8 L/kg) and higher than that of BZE (0.87 L/kg) (Mets et al. 1999). Thus, some extravascular distribution of EME might be expected in humans.

A further advantage of muscle tissue as a specimen for cocaine determination relates to post-mortem pH change in this matrix. Upon death there is a rapid decline in muscle pH as a result of post-mortem metabolism and conversion of glycogen to lactic acid. In cattle the pH has been reported to drop from pH 7.1 at the time of death to pH 5.4 – 5.7 18 – 24 hours after death and then to slowly rise again to around pH 6.5 (Lawrie and Ledward 2006).

Table 30 Cocaine and metabolite summary data in blood and thigh muscle obtained in the present study and in the published literature

			Blood concentration (mg/L)*			Thigh muscle concentration (mg/kg)			Thigh muscle/blood ratio		
			Cocaine	BZE	COET	Cocaine	BZE	COET	Cocaine	BZE	COET
[1]	9	Mean	0.049	0.33	0.015	0.12	0.44	0.026	2.9	1.7	1.5
		Mdn	0.018	0.21	0.003	0.03	0.29	0.011	2.3	1.5	1.7
		SD	0.066	0.36	0.022	0.16	0.47	0.037	0.99	0.9	0.47
		Range	0-0.17	0-0.88	0-0.052	0-0.38	0-1.3	0-0.097	2.0-4.0	0.85-3.2	1.0-1.9
[2]	9	Mean	1.9	1.8		0.33	0.22		1.2	0.26	
		Mdn	0.27	1.01		0.24	0.01		0.45	0.05	
		SD	4.0	2.2		0.35	0.34		2.2	0.34	
		Range	0.1-12	0.02-6.2		0-1.1	0-0.81		0-6.5	0-0.7	
[3]	5	Mean	11			28 (n=3)			2.4		
		Mdn	6.9			30			2.3		
		SD	12			21			0.92		
		Range	3.9-31			6.1-48			1.5-3.4		

[1] Present study; [2] Garriott 1991; [3] Poklis et al. 1987

* Garriott (1991) utilised cardiac blood; Poklis et al. (1987) did not specify the site of blood collection

Mdn: median; SD: standard deviation

The pH of intact Sikah deer muscle stored at 25°C rose from 5.7 (as measured 24 hours following death) to 6.2 in 7 days (Chapter 4). The situation in human skeletal muscle appears to be similar with early post-mortem pH values ranging from 5.7 – 6.0, depending on the specific muscle (Langford et al. 1998). Slightly higher pH values were recorded in cases with an extended post-mortem interval, particularly if the body had not been refrigerated. Post-mortem pH values for thigh muscle were typically around pH 6.0 (Langford et al. 1998). Thus, in addition to the lack of esterase activity, the greater stability of cocaine in skeletal muscle tissue as compared with blood (Chapter 4; Moriya and Hashimoto 1996) can be attributed to the rapid post-mortem decline in pH. In contrast with post-mortem blood, the concentration of cocaine and its metabolites measured in muscle should resemble more closely those at the time of death.

With the exception of case 7, in which death was believed to be an acute event following drug intake, the concentration of BZE exceeded that of cocaine in the muscle and blood with low mean cocaine/BZE ratios measured in FB (0.07; range: 0.02 – 0.1), ThM (0.14; 0 – 0.32) and CM (0.18; 0 – 0.47). In case 7 the concentration of cocaine exceeded that of BZE in ThM and CM with ratios of 2.7 and 2.9 respectively. In FB the concentrations of cocaine and BZE were near unity (0.97). This was also the only case in which the concentration of BZE was higher in the FB than it was in the muscle, implying insufficient time both for substantial enzymatic breakdown of BZE in the blood and spontaneous breakdown of cocaine to BZE in the muscle. Brain is considered an excellent specimen in the determination of cocaine. Like muscle, brain lacks esterase activity and the stability of cocaine and COET is greatly increased in this lipid-rich matrix (Moriya and Hashimoto 1996). Owing to its lipophilicity cocaine crosses the blood-brain-barrier freely (Misra et al. 1975; Nayak et al. 1976), as does COET (Hearn et al. 1991a). Because BZE does not cross the blood-brain-barrier (Misra et al. 1975; Rowbotham et al. 1990) the concentrations of BZE measured in the brain are due solely to *in situ* formation (Spiehler and Reed 1985) and as such an estimation of the approximate interval between drug ingestion and death can be made from the cocaine/BZE ratio measured in brain (Karch 2009). Such inferences cannot be made in blood because the V_d of cocaine (1.94 – 2.4 L/kg) (Hart et al. 2000) is higher than that of BZE (0.7 L/kg) (Ambre et al. 1991) and thus most of the cocaine will reside in the tissue and most of the BZE in the blood. Although the use of thigh muscle in making

such a determination would require more data, these preliminary results suggest that it is an area worthy of further investigation.

In this study the highest concentrations of cocaine were observed in the VH. Small lipophilic compounds such as cocaine should penetrate readily into the VH via diffusion (Levine and Jufer 2008). Owing to the presence of the blood-vitreous barrier equilibrium between blood and vitreous is slower than between blood and other extracellular fluids (Levine and Jufer 2008). Following drug administration the VH concentration should therefore follow the blood concentration with a certain delay. Since only free drug is able to leave the blood and enter the VH, plasma protein binding will determine the distribution equilibrium between blood and VH (Pragst et al. 1999). The protein binding of cocaine is negligible (Isenschmid 2003) and thus significant concentrations paralleling those in the blood would be expected in the VH. In this study the concentration of cocaine in VH (mean: 0.25 mg/L; mdn: 0.27 mg/L) was found to be significantly higher than that measured in FB (mean: 0.049 mg/L; mdn: 0.018 mg/L; $p = .016$) with a correlation observed between the two matrices ($\tau = .867$, $p < .01$). This observation can in part be explained by differences in the stability of cocaine in the two matrices. Whilst cocaine is broken down rapidly via chemical and enzyme hydrolysis in blood, VH, like muscle, lacks esterase activity and thus cocaine breakdown is limited to chemical hydrolysis. The increased stability of cocaine in VH is evidenced by the extended window of detection this matrix provides compared to blood (Mackey-Bojack et al. 2000; Antonides et al. 2007). This was also observed in the present study; cocaine could be detected in the VH in case 8 but not in the blood, and in case 9, cocaine was below the limit of quantification in the blood yet quantifiable in the VH.

In the few studies that have examined a large series of cocaine related deaths, the mean concentrations of cocaine in the VH were generally comparable to, or slightly higher than, those measured in FB (Logan and Stafford 1990; Mackey-Bojack et al. 2000; Duer et al. 2006; Antonides et al. 2007). The FB and VH concentration data reported by Logan and Stafford (1990), Mackey-Bojack et al. (2000) and Duer et al. (2006) are summarised in Table 31. The results obtained in these matrices in the present study are also included for comparison. Antonides et al. (2007) reported that in cases where the concentration of

cocaine in FB was higher than that measured in the VH, cocaine intoxication was apparent just prior to death and the post-mortem interval was short. Given the apparent lag between the peak concentration of cocaine in blood and VH, the significantly higher concentration of cocaine observed in the VH compared with the blood in the present study might be interpreted as a prolonged period between drug intake and death allowing a) sufficient time for diffusion of cocaine into the VH, and; b) sufficient time for substantial breakdown of cocaine in the blood. The situation is not so straight forward however, since the same observation might also reflect repeated dosing prior to death and subsequent accumulation of cocaine in the VH. In this study, death in case 7 was thought to be fairly rapid following cocaine intake and whilst this is supported by the concentration of cocaine being more than double that of the BZE in the VH, ThM and CM (cocaine/BZE ratios of 2.2, 2.7 and 2.9 respectively) the concentration of cocaine in the VH (0.46 mg/L) greatly exceeded that in the FB (0.17 mg/L) as did the concentrations measured in ThM (0.38 mg/kg) and CM (0.43 mg/kg) (concentration data tabulated in Appendix 30). The most likely explanation would be an accumulation of cocaine in the VH and muscle occurring as a result of repeated dosing leading up to death with a final dose close to the time of death as reflected by the high cocaine/BZE ratios in muscle and VH.

With the exception of case 7, the BZE concentration in the VH exceeded that of cocaine. The mean cocaine/BZE ratio in VH (excluding case 7, $n = 8$) was 0.52 (mdn: 0.50; range: 0.32 – 0.82). Because passage through the blood-vitreous barrier is proportional to lipophilicity (Barza 1981), BZE would encounter greater difficulty than cocaine in crossing the blood–vitreous barrier (Levine and Jufer 2008). In addition, the increased plasma protein binding for BZE as compared with cocaine (Isenschmid 2003) further restricts its passage into the VH. The presence of BZE in the VH might, therefore, be predominantly attributed to *in situ* chemical hydrolysis which would occur given the *in vivo* pH of VH (~pH 7.5) (Levine and Jufer 2008). A low cocaine/BZE ratio can likely be associated with a delay between cocaine administration and death. Conversely, rapid deaths might be characterised by a high cocaine/BZE ratio, as observed in case 7 (cocaine/BZE ratio: 2.2).

Previous studies have noted a much lower mean BZE concentration in VH as compared with blood (Mackey-Bojack et al. 2000; Duer et al. 2006; Antonides et al. 2007). Mackey-

Bojack et al. (2000) reported the difference between the mean BZE concentration in VH (0.99 mg/L) and FB (1.9 mg/L) to be significant (Table 31). Whilst this might be expected given the metabolites restricted entry into the VH, it is in contrast to the results presented here in which the mean BZE concentration in VH (0.41 mg/L; mdn: 0.33 mg/L) was higher than in FB (0.33 mg/L; mdn: 0.21 mg/L). This observation might reflect the delay between drug administration and death apparent in some of these cases. The opposite has been observed in rapid deaths. Antonides et al. (2007) noted that in cases where death was known to be acute following drug intake and where the cocaine concentration was higher in the blood compared with the VH, the corresponding BZE concentration was significantly higher in the blood, by as much as 10-fold. In the one case studied here where rapid death was suspected (case 7), VH BZE (0.21 mg/L) exceeded FB BZE (0.17 mg/L). Given the high concentration of cocaine measured in the VH (0.46 mg/L) compared with the FB (0.17 mg/L) in this case, repeated dosing prior to death seems likely and accumulation of cocaine in the VH would account for the higher BZE concentration in this matrix.

In accordance with previously published FB and VH concentration data (Mackey-Bojack et al. 2000), the concentrations of COET observed in the present study were quite low, probably because the amount of alcohol present is the rate limiting step in COET production (Karch 2009). A good correlation was observed between FB and VH COET in the present study ($r_s = .949$; $n = 5$; $p < .05$), whereas Mackey-Bojack et al. (2000) found no such correlation in 62 cocaine related deaths ($r = .433$) This difference might be explained by the low number of cases analysed in the present study and because in most cases the circumstances of death were similar (i.e. they appeared to be delayed). Whilst the findings of the present study and those of Mackey-Bojack et al. (2000) indicated both cocaine and BZE to be well correlated between FB and VH, observations made by Logan and Stafford (1990) suggest otherwise for cocaine (Table 31). These authors observed a correlation I of 0.70 for cocaine. In calculating the coefficient of determination (R^2) a value of 0.49 is obtained indicating a poor relationship since only 49% of the variation in concentration in one matrix could be accounted for by the other.

Multiple factors may affect the relative concentration of cocaine and metabolites in different matrices, particularly the length of the post-mortem interval and pattern of

previous use. For this reason the relationship observed between blood and either VH or muscle in a series of cases will depend on the case circumstances, with a poorer relationship likely to be observed in a series of cases in which the circumstances of death vary widely.

As with cocaine, the VH should provide an extended window of detection for BZE compared with blood. In the VH BZE would not undergo enzyme hydrolysis forming ecgonine as it does when blood passes through the liver. Further, the elimination of hydrophilic compounds such as BZE occurs together with the fluid streams and is slow in comparison with lipophilic molecules that can freely diffuse out of the VH (Larsen et al. 1991). The prolonged presence of BZE in VH was demonstrated in case 8, where BZE could be detected in the VH but not in the blood. In case 9 BZE was present at a higher concentration in the VH (0.054 mg/L) compared with the CB (0.017 mg/L) and was not detected at all in the FB (Appendix 30).

The overall pattern of cocaine distribution observed in this study ($VH > CM > ThM > FB \geq CB$) can only partly be explained by the stability of cocaine in the different matrices. The comparatively low concentrations measured in blood are due to the poor stability of cocaine in this matrix. Whilst the pH of muscle (pH 7.1) and VH (pH 7.5) *in vivo* is not outstandingly different, after death the pH of the VH does not decrease as substantially as it does in the muscle. The pH of post-mortem VH measured in 40 cases was approximately pH 7.0 (Antonides et al. 2007). Post-mortem hydrolysis of cocaine in the VH should therefore be more rapid than in the muscle, where pH values decline to around 6.0 within hours of death. The *in vitro* stability of cocaine was markedly less in sheep VH stored at 20°C compared with deer muscle tissue stored at 25°C (Chapter 4). Considering the substantially higher mean concentration of cocaine measured in the VH compared with ThM, cocaine stability in the two matrices is clearly not the governing factor. The observed concentrations will, in part, be a consequence of differences in the rate and extent of distribution of cocaine into ThM and VH as a result of differential blood flow to these tissues. A further explanation might relate to evidence that VH is not isolated from the effects of PMR.

Table 31 Cocaine and metabolite summary data obtained in blood and vitreous in the present study and in the previously published literature

Ref	N		FB concentration (mg/L)			VH concentration (mg/L)			VH/FB ratio			VH-FB correlation		
			Cocaine	BZE	COET	Cocaine	BZE	COET	Cocaine	BZE	COET	Cocaine	BZE	COET
[1]	9	Mean [†]	0.049	0.33	0.015	0.25	0.41	0.055	13	1.5	4.7	$r_s=.867^*$	$r_s=.982^*$	$r_s=.949^*$
		Mdn	0.018	0.21	0.003	0.27	0.33	0.041	14	1.2	3.7			
		SD	0.066	0.36	0.022	0.19	0.37	0.066	9.4	0.71	2			
		Range	0-0.17	0-0.88	0-0.052	0.004-0.53	0.011-0.96	0-0.18	2.8-27	0.94-2.7	3.4-7.0			
[2]	28	Mean	0.13	1.3 (n=5)		0.2	1.9 (n=24)		1.7	7.9 (n=5)		$R=0.70$		
		Mdn	0.1	0.95		0.14	1.3		1.5	1.70				
		SD	0.15	1		0.22	1.6		1.4	14				
		Range	0-0.5	0.13-2.8		0-0.79	0-5.2		0.1-6.0	0.63-33				
[3]	62	Mean	0.49	1.9	0.021	0.61	0.97	0.027	2.2	0.63	0.9	$r=.854^*$	$r=.763^*$	$r=.433$
		Mdn	0.10	0.82	0.00	0.25	0.45	0.00	1.2	0.60	0.68			
		SD	1.2	2.9	0.05	0.99	1.6	0.059	1.9	0.38	1.0			
		Range	0-6.3	0.1-14	0-0.3	0-4.5	0-11	0-0.31	0-7.4	0-1.8	0-3.0			
[4]	37	Mean	0.21	1.71		0.3	1.1							
		SD	0.33	1.6		0.46	1.1							
		Range	0-1.4	0-4.4		0-1.5	0-2.3							

[1] Present study; [2] Logan and Stafford 1990; [3] Mackey-Bojack et al. 2000; [4] Duer et al. 2006

[†] A femoral blood samples was unavailable in 3 cases

Mdn: median; SD: standard deviation

r_s = Spearman's Rho correlation coefficient; r = Pearson's correlation coefficient; R = correlation; * significant correlation ($p < .05$)

In a case report, Beno and Kriewall (1989) observed a 330% increase in VH cocaine concentration over a 19 hour interval. Significant increases (300%) in cocaine concentrations in vitreous humour have also been noted in dosed juvenile swine 8 hours following death (McKinney et al. 1995). In an investigation of PMR in a human decedent Hearn et al. (1991b) measured the VH concentrations in one eye just after death and in the other 18 hours later. The cocaine concentration had risen from 1.0 mg/L at the time of death to 3.5 mg/L after 18 hours. The concentration of BZE also rose, but to a lesser degree, from 1.1 to 1.7 mg/L. The corresponding femoral blood concentration for cocaine and BZE respectively were 1.8 and 3.6 at the time of death and 3.9 and 8.1 mg/L when sampled from the same vein after 20 hours. The substantially lower increase in the concentration of BZE in VH over time strengthens the hypothesis that BZE distribution into VH is limited. Although Hearn et al. (1991b) did not rule out differences caused by actual variations in drug concentration in left and right paired VH samples, comparison of concentrations measured in left and right paired VH samples in the present in general showed no remarkable difference.

The mechanism by which cocaine increases in the vitreous during the post-mortem interval has not been clarified but Skopp (2004) comments that it is thought possibly to be due to release from the ocular tissue or brain. The observation of increasing digoxin levels in the VH in a dog model was attributed to leaching from the choroid-retina, where the highest digoxin concentrations were measured (Binnion and Frazer 1980). An increase in MDMA concentrations in the VH in a rabbit model has been attributed to the release of drug sequestered in the globe wall (De Letter et al. 2000). Continued post-mortem diffusion of cocaine into the VH might also explain why the mean cocaine/BZE ratio measured in VH in the present study was significantly higher than in ThM (excluding case 7). The dispute over the interpretive value of VH may be largely due to continuing diffusion of cocaine into this matrix after death and it may be that the principal value of this sample for cocaine analysis is the extended period of detection it provides.

6.6 Conclusion

The findings of the present study confirm the current consensus that in cocaine related deaths isolated blood measurements are of limited interpretive value and suggest that toxicological assessments made on muscle tissue and VH offer added interpretive value. The concentration of cocaine and its metabolites measured throughout the *rectus femoris* are apparently independent of the within-muscle sampling location, reinforcing the view that this muscle could potentially provide an important specimen for interpretation in cocaine related deaths. Further, as a consequence of the greater post-mortem stability of cocaine in muscle, concentrations measured post-mortem should reflect more closely the situation at the time of death compared with blood. Muscle may also provide a far more reliable specimen than blood in cases with an extended period between death and tissue sampling, with the caveat that interpretation of results obtained in decomposing tissue would be limited owing to lack of data in such material.

Chapter 7.0 Interpretation of morphine, codeine and 6-acetylmorphine concentrations in femoral blood and vitreous humour in heroin/morphine related fatalities

7.1 Abstract

Opiates, particularly heroin, are involved in a high proportion of drug related deaths in the UK. As an aid to interpretation, the distribution of free morphine (FM), codeine and 6-acetylmorphine (6AM) in femoral blood (FB) and vitreous humour (VH) was investigated in 70 morphine involved deaths, the majority of which were heroin related. Total morphine (TM) was assayed in FB. Analytes were extracted by SPE and quantified using GC-MS/MS. Investigative reports and results of independent toxicological analyses were available. Polydrug use was highly prevalent in the cases studied. In only one case were opiates detected in the absence of another drug group. The most frequently detected other substances were alcohol (43%) and diazepam (87%). The overall distribution of FM concentrations in blood was positively skewed with 79% of cases having a FM concentration < 0.3 mg/L (median: 0.18 mg/L). Toxicological comparisons were made between rapid deaths (< 3 hours after drug intake; $n = 28$) and delayed deaths (> 3 hours; $n = 16$) ($p < .05$ considered significant). The major findings were: 1) a higher mean FM concentration in blood in rapid (0.26 mg/L) versus delayed (0.12 mg/L) deaths ($p < .001$); 2) a higher mean FM/TM ratio in rapid (0.55) versus delayed (0.26) deaths ($p < .001$); 3) a higher mean 6AM concentration in VH in rapid (0.048 mg/L) versus delayed (0.013 mg/L) deaths ($p < .01$); 4) a greater likelihood of detecting 6AM in rapid compared with delayed deaths (the detection of 6AM in blood provided a reliable indicator of rapid death), and; 5) a higher mean blood alcohol concentration and more frequent alcohol detection in rapid (mean: 67 mg/dL) versus delayed (15 mg/dL) deaths ($p < .05$).

With the exception of 6AM in delayed deaths, all opiates were well correlated between FB and VH ($r_s \geq .482$; $p < .05$). The blood detection of noscapine ($n = 10$), an opium alkaloid impurity, was associated with rapid death and significantly higher blood FM concentrations ($p < .01$) and FM/TM ratios ($p < .05$) compared to cases in which noscapine was only detectable in urine ($n = 7$). This study has demonstrated that assessments based on the

combined toxicological information obtained in FB and VH greatly assists in the interpretation of heroin-related deaths.

7.2 Introduction

The majority of drug-related deaths in the UK relate to opioid use and a high proportion of these relate specifically to heroin/morphine (Davies et al. 2010). The interpretation of heroin-related deaths is complicated because many factors affect the concentration of morphine measured at autopsy, including:

- 1) the time interval between last dose and death;
- 2) an individual's drug tolerance level;
- 3) the concomitant use of other drugs;
- 4) the post-mortem interval

Although blood is the specimen of choice for ascertaining pharmacological effects, it is sometimes unavailable at autopsy whereas vitreous humour (VH) usually is available. The VH has several advantages as a toxicological specimen. Owing to its protected environment inside the eye and location well away from drug reservoirs in the central viscera, drug concentrations in this matrix are less likely to be influenced by post-mortem changes than blood. Esterase activity is lacking in the VH and 6AM has been shown to persist in this matrix longer than in blood (Chapter 5) with concentrations of 6AM in VH consistently reported to exceed those in blood (Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007). Opioid concentration data in VH is still relatively limited and the assessment of the relationship with femoral blood (FB) concentration is too often based on small sample numbers or individual cases. Subsequently, there is conflicting evidence as to whether a relationship exists between the concentration of morphine in FB and VH (Oliver et al. 1977; Pragst et al. 1994; Gerostamoulos and Drummer 1997; Stephen et al. 2006; Antonides et al. 2007).

The primary aim of this study was to investigate the relationship between VH and FB opiate concentrations in heroin/morphine involved deaths ($n = 70$) and to further elucidate the interpretive value of both VH and FB with particular focus on the assessment of

survival time. A secondary aim of this study was to assess the role of concomitant drugs, specifically alcohol and diazepam, in heroin/morphine involved deaths.

7.3 Methods

All of the cases analysed in this study (n = 70) were Procurator Fiscal cases submitted for post-mortem examination and toxicological analysis at the Department of Forensic Medicine and Pathology, Dundee University (UK) between 2007 and 2010. The study was conducted in retrospect of toxicological analyses carried out at the department at the time the cases were submitted. The toxicological analyses carried out at the time of submission included broad spectrum drug screening in urine and blood with analyte confirmation and quantification in unpreserved FB either by HPLC-DAD or GC-MS. Ethanol was quantified by GC-Headspace analysis in FB, VH and urine preserved with 2% sodium fluoride (NaF). 6AM was confirmed in urine and FB but was not quantified. Total morphine was not quantified. VH (NaF/oxalate) and blood (NaF/oxalate and unpreserved) samples were stored at -18°C following the initial analyses. Toxicology reports for all cases were provided by the department to assist with case selection in the present study.

The criteria for case selection were:

- 1) the presence of morphine in blood was confirmed by the initial analysis;
- 2) a FB sample had been collected;
- 3) sufficient volume of NaF/oxalate preserved FB was available for analysis;
- 4) sufficient volume of NaF/oxalate preserved VH was available for analysis.

In the 70 cases that matched the above criteria, morphine, 6AM and codeine were quantified in FB and VH. Total morphine was only measured in FB because in most cases there was insufficient volume of VH (≤ 2 mL) available to carry out both free and total drug determinations. Owing to the typically low sample volume that was available, FB and VH samples collected from the right and left side of the body were pooled in each case. The time between initial testing of the samples (following autopsy) and the re-test (carried out for the present study) ranged from 3 – 30 months. The initial test results for FM were compared to those obtained in the re-test to identify potential stability issues in each case.

The toxicological results obtained in the present study were reported back to the Department of Forensic Medicine and Pathology (Dundee) where they were transferred into the appropriate cases files. Consequently, ethical approval was not required for this study and all subjects have been anonymised.

Review of investigative reports was performed to gather demographic information. Pathology reports, police reports and medical/drug histories were available in the majority of cases. Based on investigative reports, deaths were classified as rapid, delayed, or undetermined. Rapid deaths were characterised by death having occurred within three hours of drug intake (Garriott and Sturner 1973; Spiehler and Brown 1987). This determination was based on 1) witness reports, i.e. the person was observed to inject heroin and then collapse, or, the witness had seen the person alive within three hours of the reported time of death, and; 2) information from the scene, i.e. syringe still in the body, person still holding syringe, tourniquet around arm, syringe found under a slumped body, injection site oozing blood. Delayed deaths (> 3 hours) were determined from witness reports.

7.3.1 Sample analyses

Analyte extraction was conducted at the Department of Forensic Medicine and Pathology (Dundee). All chemicals and reagents were provided by the department and were of analytical grade. Details of drug standards are given in Section 3.9. Matrix matched calibration standards (water was used for VH) were prepared at concentrations of 0.005, 0.0075, 0.01, 0.025, 0.05, 0.1 and 0.2 mg/L for 6AM and 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/L for morphine and codeine. Low and high quality control (QC) standards were prepared in the blank matrix at concentrations of 0.0125 and 0.04 mg/L for 6AM and 0.03 and 0.3 mg/L for morphine and codeine using independently prepared standard solutions. Negative controls consisted of the blank matrix with no analyte added. Controls were run concurrently with the curves to determine the validity of the calibration. For TM analysis blood calibration standards containing only free morphine were prepared over the range 0.01 – 1.0 mg/L. QC samples containing M3G at low (0.17 mg/L) and high (0.67 mg/L) concentration were run concurrently with real samples. Total morphine results were corrected according to the mean percent hydrolysis obtained in concurrently run low and

high concentration controls, with 0.1 and 0.4 mg/L free morphine representing 100% hydrolytic efficiency.

With the exception of blank samples, 100µL of internal standard (IS) working solution, prepared to give a final concentration of 0.04 mg/L 6AM-d3 and 0.15 mg/L morphine-d3, was added to real samples, calibration and QC standards. Analyte extraction was performed by SPE with confirmation and quantification by GC-MS/MS. A full description of the sample preparation procedure and analytical methods is given in Section 3.9.

7.4 Statistical analyses

All statistical analyses and calculations were carried out using SPSS v.18. To characterise the distribution of the data, the means, medians, ranges and standard deviations are presented. Femoral blood and VH concentration differences were assessed using the related-samples Wilcoxon signed-rank test for non-parametric data. The Mann-Whitney test was used for comparison of the results obtained in rapid and delayed deaths. An alpha probability (p) < .05 was considered significant for both tests. Correlation coefficients (r_s) were determined using the Spearman's rho test and considered significant at the .05 level. For the purposes of statistical comparison and correlation analysis, concentrations that were below the limit of quantification (defined as the concentration of the lowest calibrator) were included in the data analysis.

7.5 Results

7.5.1 Comparison of initial and re-test blood free morphine concentration

The closeness of agreement between the initial test results (as reported by the Department of Forensic Medicine and Pathology) and the re-test results obtained in the present study was examined in each case by comparison of the FM concentration measured at each time. The re-test FM result for each case was compared to the initial FM result by calculation of the percentage deviation from the mean of the two results ((re-test result – initial result) / re-test result) x 100). Some variation in the FM concentrations measured in the initial test and re-test was expected because the sampling protocol utilised in the two tests was different. For the re-test left and right FB was pooled prior to sampling whereas the initial

test was conducted on either right or left FB. Paired left and right FB samples may vary with respect to drug concentration and such an occurrence could account for concentration differences observed between a pooled FB sample and a right or left sample. Further, different extraction procedures were used which could also account for some variation. For these reasons a deviation $\leq 25\%$ was considered to demonstrate a general agreement between the two results. The initial and re-test concentrations, percentage deviation, and storage period for each case are tabulated in Appendix 32. A scatter graph of the percentage deviation (in the negative and positive direction) versus storage time is displayed in Figure 23. The plot excludes three cases in which the initial test was carried out in either cardiac or subclavian blood and two further cases were excluded as the time of storage was unknown (no case files available).

From the scatter graph in Figure 23, it can be seen that in several cases the deviation between results was substantial; there were 13 cases in which the concentration measured in the initial test and re-test deviated by $> 25\%$. In five of these cases both the initial and re-test concentration was low (≤ 0.09 mg/L; maximum concentration difference: 0.066 mg/L) thus amplifying the apparent deviation. The interpretation of either test result in these cases would not have been different. The number of deviations in the negative direction (re-test result $<$ initial result; $n = 34$) and positive direction (re-test result $>$ initial result; $n = 33$) was approximately equal, although larger deviations were generally observed in the negative direction (Figure 23). This observation might imply that morphine degraded over time in some blood samples. To determine if there was a relationship between the size of the deviation and storage time a scatter graph was constructed plotting the percentage deviation, without the sign, against the time of storage (Figure 24). Although no relationship was observed ($R^2 = .0867$) it is acknowledged that some of the observed variation may be due to degradation or formation of morphine during storage. Other factors may also have influenced the observed concentrations. Overall the differences were not considered to be marked enough to invalidate any statistical significance derived from, or interpretations based on, the re-test data. The mean, median, standard deviation and range for the initial and re-test results were comparable (Table 32).

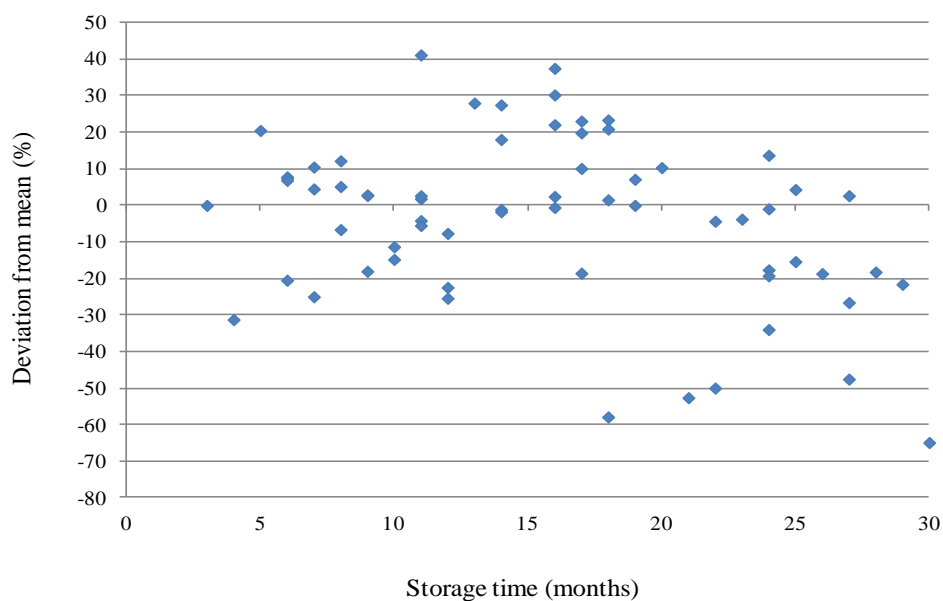


Figure 23 Scatter graph plotting the difference between the initial and re-test free morphine result in femoral blood in each case analysed (expressed as percentage deviation from the mean)

Note: A negative deviation signifies a re-test result that is lower than the initial test result.

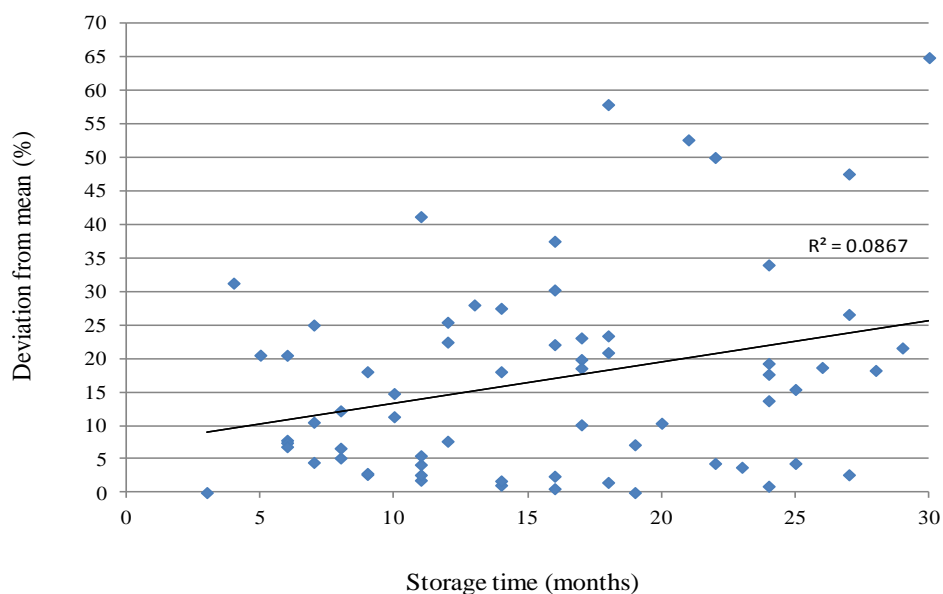


Figure 24 Scatter graph displaying the poor correlation (R^2 displayed on graph) between the time of storage and the size of the difference between initial and re-test free morphine results in femoral blood (expressed as percentage deviation from the mean)

Table 32 Initial test and re-test results summary for free morphine in blood in all cases (n = 70)

	Free morphine in femoral blood* (mg/L)	
	Initial result	Re-test result
Mean	0.29	0.27
Mdn	0.18	0.18
SD	0.54	0.57
Range	0 – 4.4	0 – 4.8

Mdn: median; SD: standard deviation

* In three cases analysed in the initial test either cardiac or subclavian blood was assayed (see Appendix 32 for more details)

Differences in the frequency of detection of 6AM in the initial and re-test results were observed. 6AM positive results obtained in the initial and re-test analyses are indicated by detection matrix in Appendix 32. The initial test for 6AM was conducted in urine and FB. In 64 of the 70 cases a urine sample was apparently available for analysis. In three cases it was unknown from the toxicology reports whether a urine sample was available and in three cases a urine sample was not available for analysis. The re-test was carried out in FB and VH, which were available in every case. In only three cases was a positive 6AM result in the initial test associated with a negative result in the re-test. The rate of detection in the initial test was substantially lower than that observed for the re-test; in the initial test 6AM was detected in urine and blood in 26 and two cases respectively. In the re-test 6AM was detected in the VH in 57 cases and in the FB in 22 cases. Thus, in 47% (n = 33) of all cases 6AM could be detected in the VH and not in the urine. Although at first glance this finding is suggestive of a longer detection window for 6AM in VH compared with urine, the explanation more likely relates to differences in the sensitivity of the analytical methods used. The experimentally determined limit of quantification for 6AM using the GC-MS/MS method validated in the present study is 0.002 mg/L in VH and 0.003 mg/L in FB. The initial test involved only the qualitative determination of 6AM in urine and blood and the limit of detection was apparently not measured. The present findings would indicate that the sensitivity of the initial test was substantially lower than that achieved in the re-test.

The fact that in some cases 6AM could still be detected in FB and VH after more than 2 years following storage at -18°C with 2% NaF demonstrates the effectiveness of these

storage conditions and provides real case evidence in support of the *in vitro* stability findings reported in Chapter 5.

7.5.2 Demographic information and circumstances of death

Demographic and other information regarding the mode and circumstances of death and other toxicological findings provided by the Department of Forensic Medicine and Pathology (Dundee) are summarised in Table 33. The mean age of subjects was 32 years (mdn: 32) with a range of 17 – 61 (10 subjects below 25 years). The mean BMI was 25.5 (mdn: 24.4) with a range of 17.1 – 37.2. Three subjects had a BMI < 18 and 23 subjects had a BMI > 25. Demographic information was missing in two cases. BMI information was missing from an additional six cases. Post-mortem examinations were carried out 1 – 6 days from the time of receipt of the body. The post-mortem interval and the length of time the body was stored at 4°C prior to autopsy are given in Table 33.

In the majority of the cases analysed in this study death was heroin related. In 57 of the 70 cases recent heroin exposures could be distinguished by the presence of 6AM either in FB or VH. In three cases (8, 40 and 52) in which 6AM was not detected in FB or VH, the use of heroin was confirmed by the detection of 6AM in urine. In two cases (31 and 32), 6AM was not detected in any matrix but the use of heroin just prior to death was confirmed by witnesses. In 35 cases the cause of death was attributed to the adverse effects (acute and/or chronic) of heroin/morphine and one other substance, predominantly alcohol or diazepam. The adverse effects of three drugs (polydrug deaths), most frequently heroin/morphine, alcohol and diazepam, was reported as cause of death in 20 cases. Death was attributed to the adverse effects (acute and/or chronic) of heroin alone in 8 cases, although other drugs were detected in low concentration in seven of these. Two deaths involving heroin were a result of hanging. In five cases (6, 24, 25, and 40) the use of heroin prior to death was suspected but could not be confirmed by the presence of 6AM, witness reports or from information obtained at the scene. However, in all cases recent injection marks were observed at post-mortem examination and the subjects were all known long-term heroin users. It is known from the investigative reports that death was delayed in cases 6 and 40 so the absence of 6AM might be expected. Mode of death was undetermined in cases 24 and

25. None of these four individuals had been prescribed morphine and it is likely that the morphine present in these cases was a result of heroin use.

In two cases death was clearly not attributable to heroin use. In case 30 FM and TM concentrations of 4.8 and 10 mg/L respectively were measured in the FB. The deceased had been prescribed Oramorph and morphine sulphate tablets. This individual was 61 years old (the oldest subject in this study), had no history of heroin abuse and there was no evidence of recent or old injection marks. Cause of death in this case was reported as oral morphine poisoning. In case 69, the FB DHC concentration was 4.8 mg/L and the cause of death was reported as DHC overdose. A relatively high FB codeine concentration was also measured in this case (0.25 mg/L). Conjugated morphine was detected at a concentration of 0.27 mg/L in this case; free morphine was not detected. In case 7, death was attributed to the adverse effects of methadone and morphine. The FM, TM and methadone concentrations in FB were 0.12, 0.61 and 2.2 mg/L respectively. It is unclear in this case whether heroin was taken prior to death. Although a known IV heroin abuser enrolled on a methadone program, 6AM was not detected and there was no mention of recent injection sites on the autopsy report. The individual had been prescribed morphine sulphate tablets which could account for the presence of morphine in the blood.

Surprisingly, in only one case (case 38) was morphine found in the absence of another substance (excluding codeine and 6AM). The most frequently detected other substance was diazepam, which was detected in 87% of cases (n = 61). In one of these cases diazepam was present in combination with another benzodiazepine, temazepam. After diazepam, ethanol was the most frequently encountered substance, detected in 43% of cases (n = 30). In 23 cases both ethanol and diazepam were detected in combination with morphine (33% of cases). Other drugs detected were cannabis (n = 21), methadone (9), cocaine (6), paracetamol (5) citalopram (3) DHC (2) fluoxetine (2) amitriptyline (2) mirtazepine (2) MDMA (1) tramadol (1) olanzepine (1) haloperidol (1) loperamide (1) sildenafil (1). Figure 26 demonstrates the frequency of polydrug use among the cases investigated in this study.

Table 33 Demographics, toxicology, and other relevant information regarding death in 70 morphine involved cases

Case no.	Sex	Age	Wt (kg)	Ht (m)	BMI	Drug / Medical History	6AM detected*	Other substances detected**	Recent injection marks	ROA (H)	Cause of death***	Mode of death	PMI (days)	Time at 4°C (days)
1	M	43	95	1.86	27.5	Chron E, H, coc	+	E[2], Dz[1]	+	IV	H/E	R	≤ 6	~ 3
2	M	31	72	1.8	22.2	Chron H + Dz / hep C	+	Dz[2], silden, mirtaz	+	IV	PD	R	~ 4	~ 3.5
3	M	31	83	1.68	29.4	Chron H (IV) + E / hep C	+	E[3]	+	IV	H/E	U	≤ 3.8	~ 3.5
4	M	31	66	1.6	25.8	Chron H (IV) + Dz	+	Dz[2], can, nosc[B]	+	IV	H/Dz	R	≤ 4	3.6
5	F	41	51	1.59	20.2	Chron H (IV) / hep C	+	Dz[2], citalop (0.41 mg/L), meth	+	IV	PD	R	< 2.1	~ 2
6	M	45	49			Long history of drug use / meth P, Dz + mitraz presc	-	Dz[3], mirtaz (0.17mg/L), meth, can	+	IV	PD	D	≤ 3.2	~3
7	M	34	100	1.82	30.2	Chron H (IV)/ meth P + M sulphate presc	-	Dz[1], meth (2.23 mg/L)	-	U	M/meth	D	~ 2.1	~ 2
8	M	20	60	1.72	20.3	Not a known abuser	+	E[1], Dz[2]	-	U	PD	D	< 5.5	5
9	M	36				Chron H, coc, Dz + M / hep C, Dz + olanz presc	+	Dz[2], coc, olanz	+	IV	H/Dz	D	~ 2.4	~ 2.3
10	M	43	63	1.56	25.9	Chron E+H	+	E[2]	+	IV	H/E	R	< 2.1	~ 2
11	M	28	71	1.65	26.1	Chron H (S+IV) meth P	+	E[3], Dz[1], fluox	+	IV	H/E	D	< 2.7	~ 2.6
12	F	18	84	1.65	30.9	Chron IV abuser	+	Dz[2],	+	IV	H/Dz	D	≤ 1.2	~ 1
13	M	22	95	1.68	33.7	H, Dz, MDMA abuse. Took 40-50 Dz before death.	+	E[2], Dz[3]	-	S	PD	U	≤ 2	~ 1.7
14	M	40	126	1.84	37.2	Chron H, coc (IV) + E / meth P	+	E[2]	+	IV	H/E	R	< 5.9	~ 5.8
15	M	39	85	1.78	26.8	Chron H (IV) + E / meth P hep B+C	+	E[3], Dz[1], nosc[B]	+	IV	H/E	U	≤ 4.7	~ 4.5
16	M	25	76	1.74	25.1	Chron H, Dz + E	+	Dz[2], coc	-	U	PD(AC)	U	≤ 3.5	~ 3
17	M	17	71	1.76	22.9	H, Dz + E abuse	+	E[2], Dz[2]	-	U	PD	U	≤ 1.4	~ 1
18	M	32	70	1.80	21.6	Chron H (IV) + Dz	+	Dz[1]	+	IV	H	U	≤ 4.4	~ 4
19	M	26	95	1.71	32.5	Chron H + coc	+	Dz[1], fluox, meth	-	S	H	R	~ 1.6	~ 1.5
20	M	40	64	1.67	22.9	Chron H (IV), E + can	+	Dz[1]	+	IV	H	R	≤ 3.9	~ 3.5

Table 33 continued

Case no.	Sex	Age	Wt (kg)	Ht (m)	BMI	Drug / Medical History	6AM detected*	Other substances detected**	Recent track marks	ROA (H)	Cause of death***	Mode of death	PMI (days)	Time at 4°C (days)
21	M	24	94	1.75	30.7	H, can, amp, MDMA + coc abuser	+	E[1], Dz[3], coc	-	S	PD	U	≤ 2.4	~ 2
22						Unknown	+	Dz[2],	-	U	H/Dz	U		
23	M	21	99	1.82	29.9	E + drug abuse	+	Dz[2], can, para	+	IV	H/Dz	R	≤ 3.6	~ 3.5
24	F	17	49	1.54	20.7	H, coc, Dz, amp + can since 12 yrs old	-	Dz[3]	+	IV	M/Dz	U	≤ 2.4	~ 2
25	M	32	63	1.79	19.7	History of abuse mainly H + coc / Dz presc	-	E[1], Dz[2]	+	IV	PD	U	≤ 2.8	~ 2.5
26	M	27	95	1.75	31.0	E + Dz abuse	+	E[3], Dz[2]	-	S	PD	U	≤ 4.7	~ 4.5
27	M	41	68	1.73	22.7	Chron E + drug abuse (for 22 yrs)	+	Coc, citalop (0.37 mg/L)	+	IV+S	H/Cit	U	U	~ 1
28	M	46	123			Chron H (IV) / meth P	+	E[2], Dz[1]	+	IV	H/E(AC)	R	< 2.6	~ 2.5
29	M	28	67	1.70	23.2	Regular use of Dz, can + E	+	Dz[3], can.	-	S	H/Dz(AC)	D	< 2.1	~ 2
30	M	61				Chron E / oramorph + M sulphate presc	-	E[3], Dz[2]	-	O(M)	M	U	≤ 2.3	~ 2
31	M	36	73	1.78	23.0	Chron Dz + can + H (smoked H before death) / hep B.	-	E[3]	-	S	H/E(AC)	R	< 3.1	~ 3
32	M	42	70	1.72	23.7	Chron E + can, occasional H (smoked H before death) / epilepsy	-	E[2], MDMA (0.23 mg/L) DHC, can	+	S	PD	R	~ 3.1	~ 3
33	M	45	63	1.60	24.6	Chron E + IV drug abuse	+	E[3]	+	IV	H/E(AC)	R	< 2.1	~ 2
34	M	36				H (IV) / hep C, naltrex presc	+	Dz[1], can	+	IV	H(AC)	R	~ 2.1	~ 2
35	M	39				Chron H + E / Dz presc	+	E[3], Dz[1], can	+	IV	H/E(AC)	R	≤ 2.7	~ 2.5
36	M	41	65	1.79	20.3	H + amp (IV). Took 500 Dz whilst in police custody	+	Dz[3], can	-	O(Dz)	Dz/H	D	< 3.1	~ 3
37	M	36	94	1.69	32.9	E + drug abuser	+	E[3], Dz[1], coc	-	S	H/E	R	≤ 3.1	~ 3
38	F	21	68	1.74	22.5	H (IV) abuse	+		+	IV	H	D	< 3.3	~ 3.2
39	M	26				Recreational can, amp, MDMA, Dz, coc + H (S)	-	Dz[2], meth (0.22 mg/L)	-	U	Dz/meth	U	≤ 4.1	~ 4
40	M	35	59	1.83	17.6	Chron H (IV)	-	Dz[1], loperamide	+	IV	H(chron)	D	< 2.1	~ 2

Table 33 continued

Case no.	Sex	Age	Wt (kg)	Ht (m)	BMI	Drug / Medical History	6AM detected*	Other substances detected**	Recent track marks	ROA (H)	Cause of death***	Mode of death	PMI (days)	Time at 4°C (days)
41	M	33				Chron drug abuser / hep C	+	Dz[2], coc, meth	+	IV	H/Dz(AC)	D	< 5.1	~ 5
42	M	33				Chron E + H (IV)	+	E[3], Dz[1]	+	IV	H/E	U	≤ 3.7	~ 3
43	M	27				H, can + meth abuser / meth P	+	E[2], Dz[3], meth (0.44 mg/L)	-	U	Hanging	U	≤ 3.5	~ 3
44	F	55				Unknown	+	Dz[2]	-	U	H/Dz	U	U	~ 0.5
45	M	28	58	1.53	24.8	Chron can, occasional H (S) / meth P	+	Dz[1], can	-	S	H	D	≤ 4.3	~ 4
46	M	31	66	1.68	23.4	H (IV) abuser	+	E[3], nosc[U]	+	IV	H/E	R	≤ 6.7	~ 5.5
47	F	25	64	1.64	23.8	IV drug abuser / hep C	+	E[3], Dz[3], can, nosc[B]	+	IV	PD	R	U	~ 3
48	M	18	71	1.72	24.0	History of H, can, coc, amp + MDMA abuse	+	Dz[2], nosc[B], papaverine	-	S	H/Dz	U	≤ 6.4	~ 6
49	F	25	45	1.62	17.1	IV drug abuser	+	Dz[2], nosc[B]	+	IV	H/Dz	U	≤ 4.6	~ 4
50	M	26	82	1.77	26.2	Regular amp, can, Dz, H + E use	+	E[2], Dz[2], DHC, can, nosc[B]	+	IV	PD	R	U	~ 2.8
51	M	26				Regular H(IV), Dz + can use	+	Dz[3], can, nosc[U]	-	U	H/Dz	U	≤ 2.3	~ 2
52	M	35				History of H (IV) abuse	+	Dz[2], citalop, can, nosc[U]	+	IV	H/Dz	D	≤ 3.4	~ 3
53	M	25	71	1.71	24.3	E + Dz use in days before death	+	Dz[2], can, nosc[U]	+	IV	H/Dz	U	≤ 6.1	~ 6
54	M	19	85	1.70	29.4	History of can, Dz + H use	+	E[3], Dz[2], can	+	IV	PD	R	≤ 2.2	~ 2
55	M	32				Chron E + history of can, Dz, DHC + H(IV) use	+	E[3], Dz[2], tramadol (0.59 mg/L)	-	U	PD	U	< 2.1	~ 2
56	M	35				Chron H (IV)	+	E[3], Dz[2]	+	IV	PD	R	< 2.1	~ 2
57	M	34	99	1.80	30.6	H (IV), coc, Dz, can + steroid abuse	+	Dz[3], DHC, nosc[U]	+	IV	H/Dz	R	< 2.1	~ 2
58	M	37	81	1.66	29.4	Meth P, Hep C	+	Dz[1], temaz(1.03 mg/L), meth (1.77 mg/L), can	-	S	PD	D	≤ 3.4	~ 3
59	F	21				Amp + coc abuser, denied H use	+	Dz[2], quinine, can	+	IV	H/Dz	R	< 2.1	~ 2
60	M	32	85	1.70	29.4	Abused a variety of drugs, old track marks present	+	E,[1] Dz[2], para, nosc[U]	-	S	H/Dz	D	≤ 5	~ 4.8

Table 33 continued

Case no.	Sex	Age	Wt (kg)	Ht (m)	BMI	Drug / Medical History	6AM detected*	Other substances detected**	Recent track marks	ROA (H)	Cause of death***	Mode of death	PMI (days)	Time at 4°C (days)
61	M	28	54	1.77	17.2	Chron H+E	+	E[3], Dz[3]	+	IV	PD	U	≤ 6	~ 5.5
62	M	35	65	1.72	22.0	IV drug abuser	+	Dz[2], can, nosc[B]	+	IV	H/Dz	R	< 3.1	~ 3
63	M	34	74	1.72	25.0	E + H (IV) abuser for 8yrs, also used can + amp	+	Dz[3], para, levamisole, haloperidol, zop, nosc[B]	+	IV	PD	R	< 4.1	~ 4
64						Unknown	+	Dz[2], amp, quinine	-	U	H/Dz	U		
65	M	34	66	1.80	20.4	Regular H (IV) abuse	+	Dz[1], can, nosc[B]	+	IV	H	R	U	~ 2
66	M	40	55	1.65	20.2	History of H (S) abuse	+	Dz[2]	-	S	Hanging	R	≤ 2.1	~ 2
67	F	20	77	1.68	27.3	E, can + amp abuse; M sulphate found in flat	+	Dz[1], amitrip, para, can	-	U	H	U	≤ 1.3	~ 1
68	M	26	67	1.69	23.5	H (IV) abuse	+	Dz[1], meth (0.021 mg/L), nosc[U]	+	IV	H	U	≤ 3.4	~ 3
69	M	33	95	1.81	29.0	Chron E. Abused drugs when young / epilepsy	-	E[1], Dz[1], DHC (4.76 mg/L), amitrip	-	O(DHC)	DHC	D	≤ 4.1	~ 3.7
70	M	28	67	1.82	20.2	H (IV) abuse and other drugs	+	Dz[3], TCA, para, can, nosc[B]	+	IV	H/Dz	R	< 4.1	~ 4

*6AM detected in either FB, VH or urine

**Substances detected in blood or urine (excluding morphine and codeine) as reported in independent toxicology reports (initial test)

*** If one or two substances were believed to have caused death they are listed. If death was thought to be attributed to the effects of more than two substances the cause of death is listed as PD (polydrug death). AC (death due to acute and chronic effects)

Drug medical history: Chron (chronic use); hep (hepatitis); meth P (in methadone program); presc (current prescription)

Other substances: E (blood ethanol), E[1] (< 50 mg/dL), E[2] (50 – 100 mg/dL), E[3] (> 100 mg/dL); H (heroin); M (morphine); meth (methadone); DHC (dihydrocodeine); Dz (blood diazepam), Dz[1] (< 0.16 mg/L), Dz[2] (0.16 – 1 mg/L), Dz[3] (> 1 mg/L); temaz (temazepam); coc (cocaine); can (cannabis); MDMA (3,4-methylenedioxymethamphetamine (ecstasy)); nosc (noscipine); nosc[B]; noscapine detected in blood; nosc[U]; noscapine detected in urine only; fluox (fluoxetine); amitrip (amitriptyline); mirtaz (mirtazepine); olanz (olanzapine) citalop (citalopram); zop (zopiclone); para (paracetamol)

Route of administration: IV (intravenous); S (smoked); O (oral); U (undetermined)

Mode of death: R (rapid: < 3 hours); D (delayed: > 3 hours); U (undetermined)

The most commonly detected heroin impurity was noscapine (n = 17). Papaverine, also an impurity obtained from the opium poppy, was detected in one case. Quinine, sometimes used as a cutting agent in heroin and cocaine, was found in two cases. Levamisole, widely used in veterinary medicine as a de-wormer and commonly used as an adulterant in cocaine, was detected in the blood of one heroin user.

The classification of deaths as either, rapid (< 3 hours) or delayed (> 3 hours) was based solely on investigative reports and not inferred from the toxicological results. Where the mode of death could not be confirmed from witness statements or from information at the scene, death was classified as undetermined. Rapid deaths were confirmed in 28 cases and delayed death in 16 cases. The remaining 26 cases were undetermined.

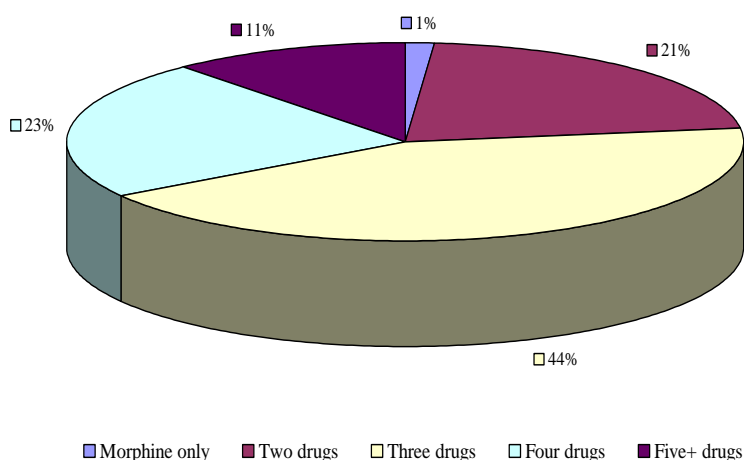


Figure 25 Frequency of polydrug use; number of drugs detected (including morphine) in 70 morphine positive cases

7.5.3 Analyte concentration in blood and vitreous humour

Concentration data in blood and vitreous in each case is tabulated in Table 34 and displayed graphically in Figure 25 (free morphine), 26 (codeine) and 27 (6AM). Analyte concentration data is summarised (mean, median, range and standard deviation) for FB (Table 35), VH (Table 35) and for VH to FB ratios (Table 37) in 3 separate groups: 1) all

cases, 2) rapid deaths, and 3) delayed deaths. Where distributions were highly skewed the median provided a more appropriate descriptive statistic than the mean. The statistical significance of the difference between rapid and delayed means is also reported in each table. Spearman's rho correlation coefficients and corresponding significance levels obtained for all cases and in rapid and delayed deaths are reported in Table 38. Correlation graphs for morphine, codeine and 6AM for all cases are displayed in Appendix 33. The significance of the difference between concentrations measured in blood and vitreous in all cases and in rapid and delayed deaths is also reported in Table 38.

7.5.3.1 Free and total morphine

The distribution of morphine concentration in blood was positively skewed, demonstrating that in the majority of cases the free morphine concentrations were at the low end of the observed range (Figure 28a). Seventy nine percent of cases had a blood morphine concentration of < 0.3 mg/L. The maximum FM concentration measured in FB was 4.8 mg/L. The overall mean was 0.27 mg/L and the median (mdn) was much lower (0.18 mg/L). When only considering rapid deaths ($n = 28$), the concentration measured in FB (mean: 0.26 mg/L; mdn: 0.28 mg/L; range: 0.069 – 0.64 mg/L) was found to be significantly higher than in delayed deaths (mean and mdn: 0.12 mg/L; range: 0 – 0.26 mg/L; $p < .001$). The difference in the distribution of morphine in rapid and delayed deaths is illustrated by the box plot in Figure 28b. The mean (and median) TM concentration was comparable in rapid (0.50 mg/L; mdn: 0.48 mg/L) and delayed deaths (0.48 mg/L; mdn: 0.44 mg/L; Table 35). In comparing the FM to TM ratio (FM/TM) in blood, a significant difference was observed between rapid and delayed deaths ($p < .001$). Rapid deaths were characterised by a higher mean FM/TM ratio (0.55) compared to delayed deaths (0.26; Table 35). Some overlap was observed; two rapid deaths had low ratios (0.19 and 0.21) and two delayed deaths had ratios of 0.51 and 0.54, and thus more characteristic of rapid death. Irrespective of mode of death, good correlations ($p < .01$) were observed between FM and TM in blood (Table 38).

Table 34 Toxicological results obtained in femoral blood and vitreous humour in 70 morphine-involved deaths

Case	Femoral blood concentration (mg/L)						Vitreous humour concentration (mg/L)			
(MOD)	FM	TM	FM/TM	6AM	Codeine	C/FM	FM (VH/FB)	6AM (VH/FB)	Codeine (VH/FB)	C/FM
1 (R)	0.29	0.32	0.90	0.008	0.021	0.07	0.48 (1.7)	0.099 (13)	0.054 (2.6)	0.11
2 (R)	0.20	0.38	0.52	0.004	0.021	0.11	0.12 (0.62)	0.042 (11)	0.018 (0.86)	0.15
3 (U)	0.15	0.15	1.04	0.007	0.015	0.10	0.11 (0.75)	0.049 (7.5)	0.016 (1.1)	0.14
4 (R)	0.40	0.57	0.69	0.008	0.042	0.11	0.20 (0.50)	0.069 (8.6)	0.038 (0.90)	0.19
5 (R)	0.38	0.49	0.77	0.019	0.030	0.08	0.20 (0.52)	0.15 (8.0)	0.027 (0.90)	0.14
6 (D)	0.095	0.34	0.28	0	0.010	0.11	0.20 (2.2)	0	< 0.01	
7 (D)	0.12	0.66	0.17	0	< 0.01		0.20 (1.8)	0	< 0.01	
8 (D)	0.018	0.093	0.19	0	< 0.01		< 0.01	0	< 0.01	
9 (D)	0.024	0.20	0.12	0	0.014	0.58	0.12 (5.2)	0.006	0.014 (1.0)	0.11
10 (R)	0.30	0.61	0.49	0.007	0.031	0.10	0.097 (0.33)	0.048 (6.5)	0.010 (0.32)	0.10
11 (D)	0.24	0.47	0.51	< 0.005	0.036	0.15	0.41 (1.7)	0.028	0.078 (2.2)	0.19
12 (D)	0.14	0.43	0.32	0	0.020	0.14	0.032 (0.23)	0.006	0.014 (0.70)	0.44
13 (U)	0.083	0.11	0.73	< 0.005	0.016	0.19	0.046 (0.55)	0	0.012 (0.75)	0.26
14 (R)	0.22	0.28	0.77	0	0.028	0.13	0.20 (0.93)	0.017	0.029 (1.0)	0.15
15 (U)	0.15	0.44	0.34	0	0.022	0.15	0.20 (1.3)	0.041	0.023 (1.1)	0.12
16 (U)	0.12	0.37	0.33	0	0.023	0.19	0.12 (0.99)	0.013	0.047 (2.0)	0.39
17 (U)	0.22	NA		0	0.036	0.16	0.21 (0.94)	0.033	0.071 (2.0)	0.34
18 (U)	0.16	1.1	0.15	0	0.031	0.19	0.12 (0.74)	0.009	0.037 (1.2)	0.31
19 (R)	0.31	0.85	0.37	0	0.047	0.15	0.21 (0.66)	0.023	0.070 (1.5)	0.34

Table 34 continued

Case	Femoral blood concentration (mg/L)						Vitreous humour concentration (mg/L)			
(MOD)	FM	TM	FM/TM	6AM	Codeine	C/FM	FM (VH/FB)	6AM (VH/FB)	Codeine (VH/FB)	C/FM
20 (R)	0.40	0.74	0.54	< 0.005	0.038	0.10	0.40 (1.0)	0.059	0.056 (1.5)	0.14
21 (U)	0.083	0.33	0.25	0	0.024	0.29	0.035 (0.42)	0.007	0.018 (0.75)	0.51
22 (U)	0.18	1.0	0.18	0	0.026	0.14	0.055 (0.30)	< 0.005	0.021 (0.81)	0.38
23 (R)	0.13	0.34	0.38	0	0.025	0.19	0.11 (0.84)	< 0.005	0.033 (1.3)	0.31
24 (U)	0.83	1.6	0.52	0	0.14	0.17	0.61 (0.73)	0	0.16 (1.1)	0.25
25 (U)	0.087	0.16	0.54	0	0.021	0.24	0.058 (0.67)	0	0.020 (0.95)	0.34
26 (U)	0.064	0.17	0.37	0	0.013	0.20	0.029 (0.45)	< 0.005	0.019 (1.5)	0.66
27 (U)	0.12	0.63	0.19	< 0.005	0.045	0.39	0.055 (0.47)	< 0.005	0.026 (0.58)	0.47
28 (R)	0.28	0.50	0.56	0	0.032	0.11	0.16 (0.58)	0.036	0.044 (1.4)	0.27
29 (D)	0.088	0.45	0.20	0	0.022	0.25	0.034 (0.39)	< 0.005	0.034 (1.6)	1.00
30 (U)	4.8	10.0	0.49	0	0.039	0.01	0.42 (0.09)	0	0.058 (1.5)	0.14
31 (R)	0.069	0.095	0.73	0	0.012	0.17	0.087 (1.3)	0	0.033 (2.8)	0.38
32 (R)	0.11	0.27	0.41	0	0.027	0.24	0.13 (1.1)	0	0.074 (2.7)	0.58
33 (R)	0.16	0.76	0.21	0.006	0.026	0.17	0.12 (0.74)	0.045 (7.5)	0.031 (1.2)	0.27
34 (R)	0.18	0.48	0.37	0	0.036	0.20	0.092 (0.52)	0.03	0.024 (0.67)	0.26
35 (R)	0.22	0.36	0.62	0	0.028	0.13	0.073 (0.33)	0.022	0.024 (0.86)	0.33
36 (D)	0.20	0.38	0.54	0	0.028	0.14	0.063 (0.31)	0.007	0.017 (0.61)	0.27
37 (R)	0.11	0.28	0.39	0	0.019	0.17	0.079 (0.72)	0.012	0.025 (1.3)	0.32
38 (D)	0.21	1.2	0.18	0	0.018	0.09	0.21 (0.98)	0.032	0.058 (3.2)	0.28
39 (U)	< 0.01	0.019		0	< 0.01		< 0.01	0	< 0.01	

Table 34 continued

Case	Femoral blood concentration (mg/L)						Vitreous humour concentration (mg/L)			
(MOD)	FM	TM	FM/TM	6AM	Codeine	C/FM	FM (VH/FB)	6AM (VH/FB)	Codeine (VH/FB)	C/FM
40 (D)	< 0.01	0.036		0	0		0.044	0	< 0.01	
41 (D)	0.16	0.89	0.18	0	0.049	0.31	0.15 (0.91)	0.01	0.086 (1.8)	0.59
42(U)	0.47	1.1	0.42	< 0.005	0.083	0.18	0.48 (1.0)	0.091	0.14 (1.6)	0.28
43 (U)	0.040	NA		0	< 0.01		0.047 (1.2)	< 0.005	0.037	0.79
44 (U)	0.024	0.32	0.08	0	< 0.01		0.032 (1.3)	< 0.005	0.014	0.44
45 (D)	0.12	0.68	0.18	< 0.005	0.018	0.15	0.028 (0.23)	0.009	0.016 (0.89)	0.57
46 (R)	0.27	0.48	0.56	0.009	0.026	0.10	0.14 (0.51)	0.028 (3.1)	0.026 (1.0)	0.19
47 (R)	0.38	0.65	0.59	< 0.005	0.034	0.09	0.24 (0.62)	0.02	0.024 (0.71)	0.10
48 (U)	0.33	0.54	0.61	0	0.040	0.12	0.33 (0.99)	0.024	0.054 (1.4)	0.17
49 (U)	0.89	1.1	0.78	0	0.050	0.06	1.1 (1.2)	0.04	0.17 (3.5)	0.16
50 (R)	0.29	0.46	0.64	0	0.028	0.10	0.24 (0.83)	0.028	0.049 (1.8)	0.20
51 (U)	0.22	0.78	0.29	0	0.029	0.13	0.055 (0.25)	< 0.005	0.019 (0.66)	0.35
52 (D)	0.26	0.74	0.35	0	0.030	0.12	0.34 (1.3)	0	0.053 (1.8)	0.15
53 (U)	0.22	1.1	0.21	0	0.038	0.17	0.26 (1.1)	0.013	0.10 (2.7)	0.40
54 (R)	0.088	0.46	0.19	0	0.019	0.22	0.046 (0.52)	0.005	0.023 (1.2)	0.50
55 (U)	0.063	0.20	0.31	0	< 0.01		0.056 (0.89)	0.017	0.032	0.57
56 (R)	0.38	0.54	0.69	< 0.005	0.032	0.09	0.57 (1.5)	< 0.005	0.11 (3.5)	0.20
57 (R)	0.095	0.29	0.33	0	0.013	0.14	0.048 (0.51)	0.02	0.019 (1.5)	0.40
58 (D)	0.072	0.26	0.28	0	0.011	0.15	0.3 (4.1)	0	0.067 (6.1)	0.23

Table 34 continued

Case	Femoral blood concentration (mg/L)						Vitreous humour concentration (mg/L)			
(MOD)	FM	TM	FM/TM	6AM	Codeine	C/FM	FM (VH/FB)	6AM (VH/FB)	Codeine (VH/FB)	C/FM
59 (R)	0.11	0.18	0.65	0	0.013	0.12	0.10 (0.92)	< 0.005	0.014 (1.1)	0.13
60 (D)	0.21	0.54	0.39	0	0.028	0.13	0.16 (0.77)	0.02	0.043 (1.5)	0.27
61 (U)	0.18	0.83	0.21	< 0.005	0.027	0.15	0.15 (0.83)	0.012	0.045 (1.7)	0.31
62 (R)	0.38	0.55	0.68	< 0.005	0.026	0.07	0.33 (0.88)	0.11	0.065 (2.5)	0.20
63 (R)	0.25	0.35	0.72	< 0.005	0.021	0.08	0.13 (0.49)	0.039	0.023 (1.1)	0.18
64 (U)	0.022	0.079	0.28	0	< 0.01		0.028 (1.3)	0.006	0.018	0.64
65 (R)	0.38	0.76	0.50	< 0.005	0.025	0.07	0.12 (0.31)	0.068	0.03 (1.2)	0.25
66 (R)	0.33	0.85	0.38	0.009	0.051	0.16	0.20 (0.61)	0.13 (15)	0.051 (1.0)	0.26
67 (U)	0.20	1.0	0.19	0	0	0	0.15 (0.76)	0.01	0.012	0.08
68 (U)	0.011	0.013	0.85	0	0	0	0.029 (2.6)	0.005	0.011	0.38
69 (D)	0	0.27	0	0	0.25		0.024	0	0.022 (0.09)	0.92
70 (R)	0.64	1.0	0.62	0.014	0.13	0.20	0.54 (0.84)	0.13 (9.1)	0.17 (1.4)	0.32

MOD: mode of death (R: rapid, D: delayed, U: undetermined); NA: not analysed

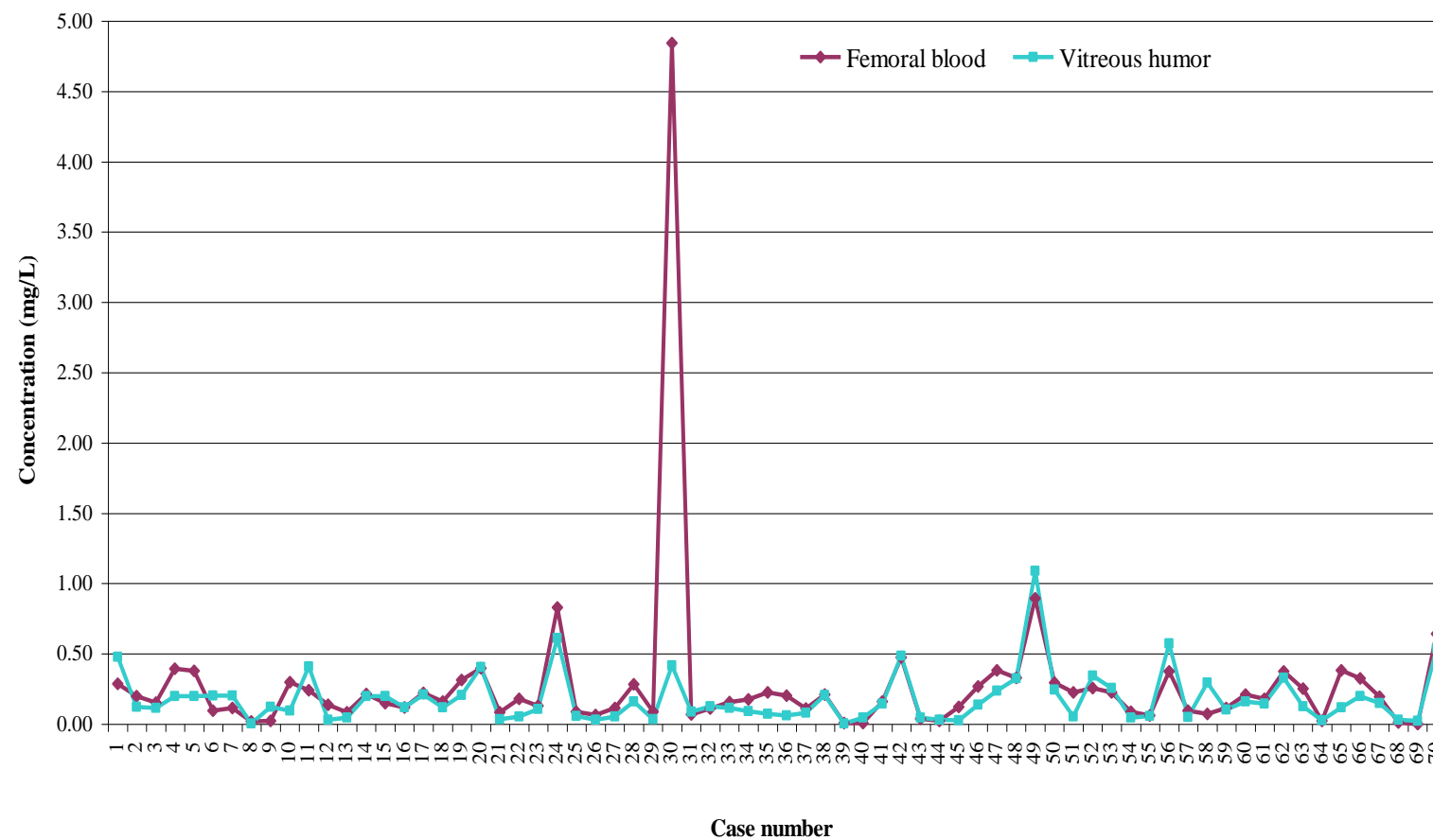


Figure 26 Free morphine measured in femoral blood and vitreous humour in 70 cases

Concentrations represent the mean of duplicate extractions

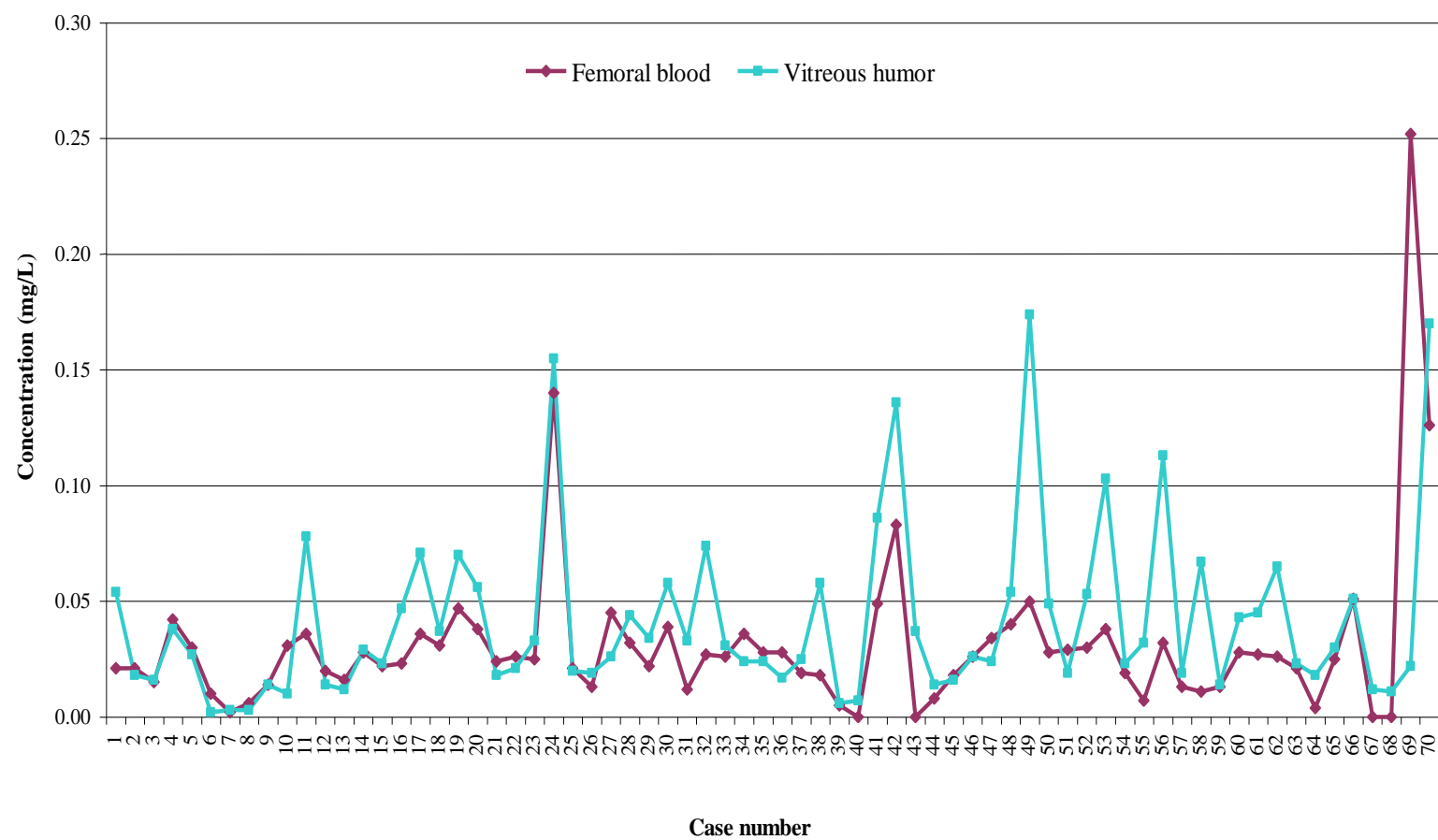


Figure 27 Codeine measured in femoral blood and vitreous humour in 70 morphine positive cases
Concentrations represent the mean of duplicate extractions

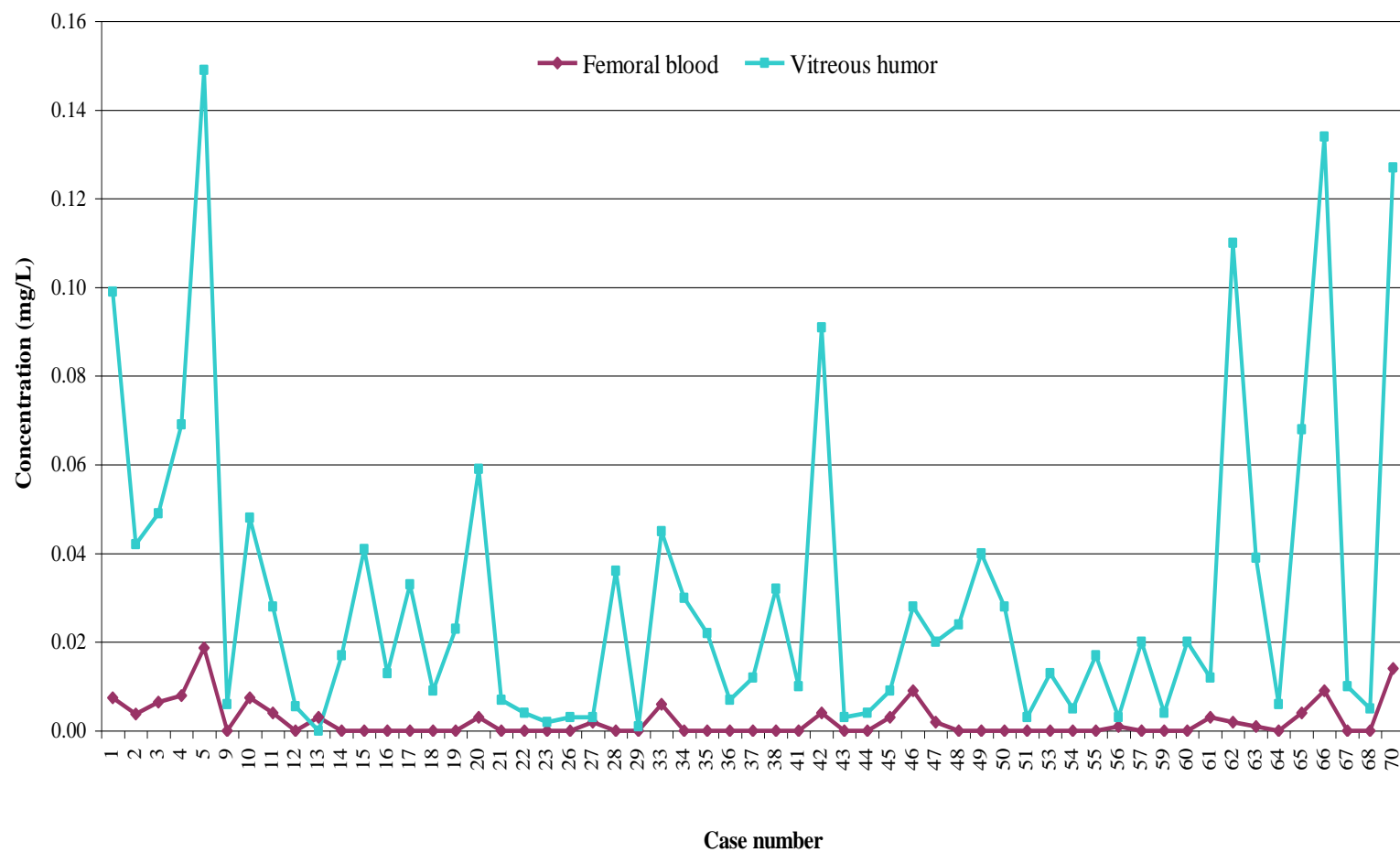


Figure 28 6AM measured in femoral blood and vitreous humour in 57 6AM positive cases

Concentrations represent the mean of duplicate extractions

The concentration of FM in the VH (mean: 0.18 mg/L; mdn: 0.12 mg/L) was found to be significantly lower than in FB (mean: 0.27 mg/L; mdn: 0.18 mg/L; $p = .001$; Table 38). In rapid deaths, the concentration of FM in the VH (mean: 0.20 mg/L; mdn: 0.13 mg/L) was also significantly lower than that measured in FB (mean: 0.26 mg/L; mdn: 0.28 mg/L; $p < .001$; Table 38). In delayed deaths, the mean FM concentration in VH (0.15 mg/L) was slightly higher than the corresponding result for FB (0.12 mg/L) but the difference between groups was not significant ($p > .05$). Thus, the VH/FB morphine concentration ratio in rapid deaths (mean: 0.75; mdn: 0.64) was typically lower than in delayed deaths (mean: 1.7; mdn: 0.98), although again the difference was not significant ($p > .05$; Table 37). The median ratio in delayed deaths (0.98) was much lower than the mean (1.7) and reflects the greater positive skew in the distribution of this data set. The difference between the VH morphine concentration measured in rapid and delayed deaths was not significant ($p > .05$; Table 36). A correlation was observed between FB and VH morphine ($r_s = .782$; $p < .01$; $n = 70$; Table 38; Appendix 33). The correlation obtained when only rapid deaths were included ($r_s = .756$; $n = 28$) was better than that obtained for delayed deaths ($r_s = .600$; $n = 16$; Appendix 34) although in both groups the relationship was significant ($p < .01$).

7.5.3.2 Codeine

The maximum codeine concentration measured in FB was 0.25 mg/L with an overall mean of 0.031 mg/L (mdn: 0.026 mg/L; Table 35). In VH the maximum was 0.17 mg/L with a mean of 0.041 mg/L (mdn: 0.028 mg/L; Table 36). The FB codeine concentration measured in rapid deaths (mean: 0.031 mg/L; mdn: 0.028 mg/L) was found to be significantly higher than that measured in delayed deaths (mean: 0.034 mg/L; mdn: 0.019 mg/L; $p = .030$; Table 35). In VH, the difference between the concentration of codeine in rapid (mean: 0.043 mg/L; mdn: 0.031 mg/L) and delayed deaths (mean: 0.032 mg/L; mdn: 0.020 mg/L) was not significant ($p > .05$; Table 36). Although the mean codeine concentration measured in VH in all cases (0.041 mg/L) was only marginally higher than the corresponding result for FB (0.031 mg/L), and with comparable medians (VH: 0.028 mg/L; FB: 0.026 mg/L), statistical comparison of the two groups revealed that the difference was significant ($p < .001$; Table 38). A significantly higher codeine concentration in the VH (mean: 0.043 mg/L) compared with the FB (mean: 0.031 mg/L) was also observed in rapid deaths ($p = .003$; Table 38). There was no statistical difference between the concentration of codeine in

FB (mean: 0.034 mg/L; mdn: 0.019 mg/L) and VH (mean: 0.032 mg/L; mdn: 0.020 mg/L) in delayed deaths ($p > .05$; Table 38). The mean VH to FB concentration ratio for codeine was 1.5 overall, 1.4 in rapid deaths, and 1.6 in delayed deaths (Table 37). Codeine was found to be correlated between FB and VH in all cases ($r_s = .672$; $p < .01$; $n = 70$) and in rapid deaths ($r_s = .482$; $p < .01$; $n = 28$) but not in delayed deaths $p > .05$; $n = 16$; Table 38). A scatter graphs illustrating the correlation between FB and VH for codeine in all cases is displayed in Appendix 33.

The codeine/free morphine (C/FM) ratio measured in VH (mean: 0.35) was significantly higher than in FB (mean: 0.16; $p < .001$; Table 38). The mean C/FM ratio measured in FB and VH in rapid deaths (FB: 0.13; VH: 0.25) was lower than the corresponding ratio in delayed deaths (FB: 0.17; VH: 0.37) although statistical comparison of C/FM ratios in FB and VH did not reveal any statistical difference ($p > .05$). When considering only cases in which heroin exposure was confirmed by the presence of 6AM in any matrix, or from witness reports ($n = 62$), the mean C/FM ratio in FB and VH was 0.16 and 0.32 respectively, and thus comparable to the mean obtained when considering all cases. A correlation was observed between the C/FM ratios measured in FB and VH when including all cases ($r_s = .658$; $p < .01$; Table 38) and when considering only rapid deaths ($r_s = .709$; $p < .01$). In the delayed death group, case 9 prevented a significant correlation being obtained ($r_s = .489$; $p = > .05$). In this case the morphine concentration was much higher in the VH (0.12 mg/L) compared with the FB (0.024 mg/L), yet the corresponding codeine concentrations were equal in both matrices (0.014 mg/L). With the exclusion of case 9 from the delayed death group, a correlation coefficient of .717 was obtained ($p < .01$).

7.5.3.3 6AM

Summary and statistical data for 6AM was calculated for cases in which the analyte was detected in blood and/or VH ($n = 57$). 6AM was detected in the VH in 56 cases and in both the blood and VH in 21 cases. In one case (case 13) 6AM was detected in the blood and not in the VH. Of the 6AM positive cases, 26 were rapid deaths, ten were delayed and in 22 cases the mode of death was undetermined. Of the 22 cases in which 6AM was detected in blood, 15 were rapid deaths, two were delayed and the remainder undetermined. The mean 6AM concentration in FB was 0.002 mg/L, with a maximum of 0.019 mg/L (Table 35).

Table 35 Femoral blood concentration summary in all cases and in deaths grouped by mode of death with rapid versus delayed group comparisons

			Overall (n =70)	Rapid (n =28)	Delayed (n = 16)	Rapid-delayed group comparison		
						<i>U</i>	<i>z</i> -score	Sig (<i>p</i>)
FB	FM (mg/L)	Mean	0.27	0.26	0.12	87.00	-3.343	< .001*
		Mdn	0.18	0.28	0.12			
		Range	0 – 4.8	0.069 – 0.64	0 – 0.26			
		SD	0.58	0.13	0.085			
	TM (mg/L)	Mean	0.66 (n=68)	0.50	0.48	198.00	-.634	.269
		Mdn	0.46	0.48	0.44			
		Range	0.013 – 10	0.095 – 1.0	0.036 – 1.2			
		SD	0.93	0.22	0.30			
	FM/TM	Mean	0.43 (n=68)	0.55	0.26	45.00	-4.367	< .001*
		Mdn	0.39	0.56	0.22			
		Range	0 – 1.0	0.19 – 0.90	0 – 0.54			
		SD	0.22	0.18	0.14			
	6AM (mg/L)	Mean	0.002 (n = 57)	0.004 (n=26)	0.001 (n = 10)	71.50	-1.848	.030*
		Mdn	0	0.002	0			
		Range	0 – 0.019	0 – 0.019	0 – 0.004			
		SD	0.004	0.005	0.002			
	Codeine (mg/L)	Mean	0.031	0.031	0.034	147.00	-1.881	.030*
		Mdn	0.026	0.028	0.019			
		Range	0 – 0.25	0.012 – 0.13	0 – 0.25			
		SD	0.035	0.021	0.060			
	C/FM	Mean	0.16	0.13	0.17	174.00	-.917	.185
		Mdn	0.14	0.11	0.14			
		Range	0 – 0.63	0.07 – 0.24	0 – 0.58			
		SD	0.11	0.049	0.14			

Mdn: median; SD: standard deviation; * Significant Mann-Whitney test result ($p < .05$ for a 1-tailed test)

Table 36 Vitreous humour concentration summary in all cases and in deaths grouped by mode of death with rapid versus delayed group comparisons

Matrix	Analyte		Overall (n =70)	Rapid (n =28)	Delayed (n = 16)	Rapid-delayed group comparison		
						<i>U</i>	<i>z</i> -score	Sig (<i>p</i>)
VH	FM (mg/L)	Mean	0.18	0.20	0.15	176.50	-1.159	.126
		Mdn	0.12	0.13	0.14			
		Range	0.004 – 1.1	0.046 – 0.57	0.004 – 0.41			
		SD	0.18	0.14	0.13			
	6AM (mg/L)	Mean	0.031 (n = 57)	0.048 (n = 26)	0.013 (n = 10)	51.00	-2.493	.006*
		Mdn	0.020	0.033	0.009			
		Range	0 – 0.15	0.002 – 0.15	0.001 – 0.032			
		SD	0.035	0.043	0.011			
	Codeine (mg/L)	Mean	0.041	0.043	0.032	166.00	-1.416	.080
		Mdn	0.028	0.031	0.020			
		Range	0.002 – 0.17	0.010 – 0.17	0.002 – 0.086			
		SD	0.038	0.033	0.028			
	C/FM	Mean	0.35	0.25	0.37	187.00	-.903	.189
		Mdn	0.28	0.23	0.27			
		Range	0.01 – 1.5	0.10 – 0.58	0.01 – 1.0			
		SD	0.28	0.12	0.31			

Mdn: median; SD: standard deviation;

* Significant Mann-Whitney test result ($p < .05$ for a 1-tailed test)

Table 37 Vitreous humour to femoral blood concentration ratio summary for free morphine, 6AM and codeine in all cases and in deaths grouped by mode of death with rapid versus delayed group comparisons

Matrix	Analyte		Overall (n =70)	Rapid (n =28)	Delayed (n = 16)	Rapid-delayed group comparison		
						<i>U</i>	<i>z</i> -score	Sig (<i>p</i>)
VH/FB	FM	Mean	0.99	0.75	1.7	162.00	-1.223	.113
		Mdn	0.76	0.64	0.98			
		Range	0.22 – 5.2	0.31 – 1.7	0.23 – 5.2			
		SD	0.93	0.34	1.7			
	6AM	Mean	12 (n=57)	15 (n=26)	5.0 (n=10)	3.50	-1.715	.051
		Mdn	8.3	10	5.0			
		Range	0 – 55.0	3.0 – 55	3.0 – 7.0			
		SD	13	14	2.8			
	Codeine	Mean	1.5	1.4	1.6	200.00	-.255	.403
		Mdn	1.2	1.2	1.0			
		Range	0.09 – 6.1	0.32 – 3.5	0.09 – 6.1			
		SD	1.1	0.74	1.5			

Mdn: median; SD: standard deviation;

* Significant Mann-Whitney test result ($p < .05$ for a 1-tailed test)

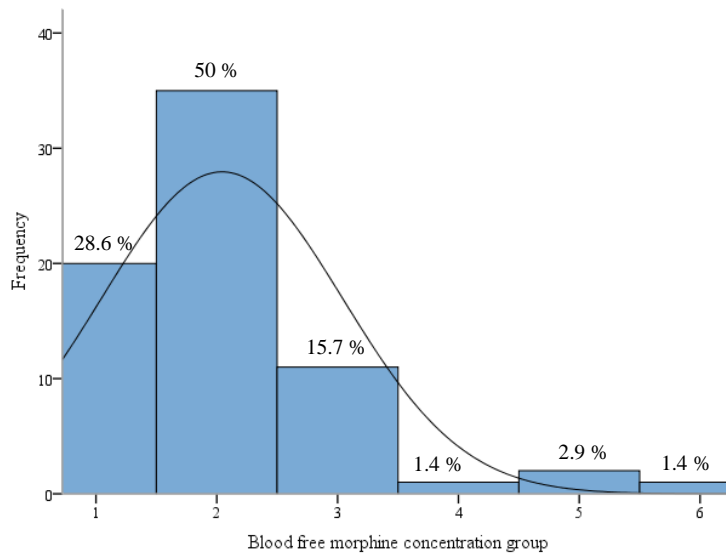
Table 38 Femoral blood and vitreous correlations and matrix comparisons by mode of death and with inclusion of all cases

		All data (n = 70)	Rapid (n=28)	Delayed (n = 16)
<u>FM (VH-FB)</u>				
<i>Correlation</i>	r_s	.782	.756	.600
	Sig. level	.01	.01	.01
<i>Group comparison</i>	<i>z-score</i>	-3.213	-3.279	-0.672
	<i>Sig (p)</i>	.001*	.000*	.260
<u>6AM (VH-FB) [†]</u>				
<i>Correlation</i>	r_s	.604 (n=57)	.783 (n=26)	.156 (n=10)
	Sig. level	.01	.01	> .05
<i>Group comparison</i>	<i>z-score</i>	-6.512 (n = 57)	-4.458 (n = 26)	-2.807 (n = 10)
	<i>Sig (p)</i>	< .001*	< .001*	.001*
<u>Codeine (VH-FB)</u>				
<i>Correlation</i>	r_s	.672	.482	.695
	Sig. level	.01	.01	.01
<i>Group comparison</i>	<i>-z-score</i>	-4.230	-2.643	-1.433
	<i>Sig (p)</i>	< .001*	.003*	.084
<u>C/FM (VH-FB)</u>				
<i>Correlation</i>	r_s	.658	.709	.489
	Sig. level	.01	.01	> .05
<i>Group comparison</i>	<i>z-score</i>	-6.640	-4.600	-2.329
	<i>Sig (p)</i>	< .001*	.000*	.009*
<u>FM-TM (FB)</u>				
<i>Correlation</i>	r_s	.747	.773	.774
	Sig. level	.01	.01	.01

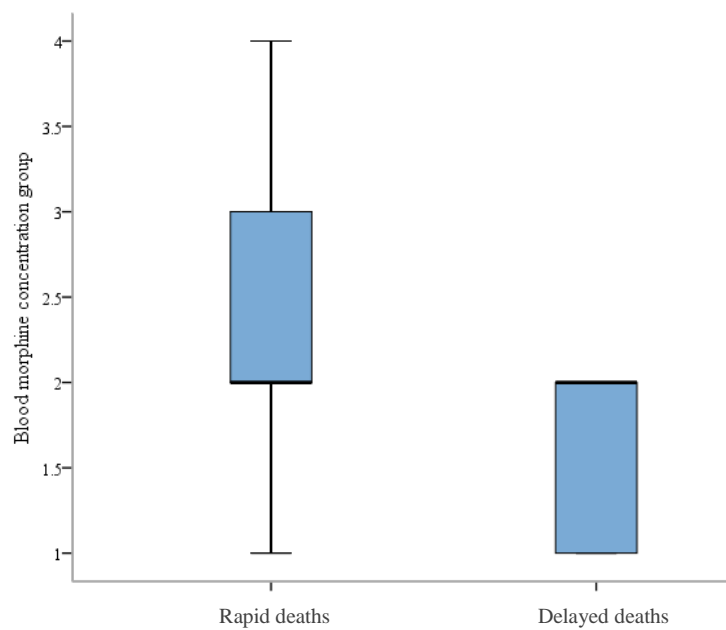
r_s : Spearman's correlation coefficient (Sig. level < .05 significant for a 1-tailed test)

[†] Only cases in which 6AM was detected in VH and/or FB are included

*Significant Wilcoxon signed ranks test result ($p < .05$ for a 1-tailed test)



(A)



(B)

Concentration group (mg/L): [1] < 0.1; [2] 0.1 – 0.3; [3] 0.3 – 0.5 [4] 0.5 – 0.7; [5] 0.7 – 0.9 [6] > 0.9

Figure 29 Distribution frequency (with standard error) of blood free morphine concentration measured in (A) all morphine involved deaths (n = 70) and (B) rapid (n = 28) and delayed (n = 16) deaths

In VH the mean 6AM concentration (0.031 mg/L; Table 36) was significantly higher than in the blood ($p < .001$; Table 38) and the maximum concentration measured was 0.15 mg/L. When considering only rapid deaths ($n = 26$) the mean 6AM concentration in FB and VH was 0.004 and 0.048 mg/L respectively. In delayed deaths ($n = 10$) the 6AM concentration in VH (mean: 0.013 mg/L) was significantly lower than in rapid deaths ($p = .006$; Table 36). In two delayed deaths 6AM was detected in the blood at a concentration below the limit of quantification (< 0.005 mg/L).

7.5.3.4 Morphine and 6AM in noscapine positive cases

Noscapine was detected in blood and/or urine in 17 6AM positive cases. In ten cases noscapine was detectable in blood and in seven cases it could only be detected in the urine. In two of the noscapine positive cases death was known to be delayed and in both of these cases noscapine was detected only in urine and 6AM was detected only in the VH. Of the ten blood positive cases, the mode of death was undetermined in two and rapid in eight with 6AM detected in the blood in six of the rapid cases. Statistical comparisons for morphine (free and total) and 6AM were carried out between cases in which noscapine could be detected in blood and cases where it was only detected in urine (Table 39). In blood positive noscapine cases the mean concentration of 6AM in the VH (0.057 mg/L; $n = 10$) was higher than the overall mean for VH (0.031 mg/L; $n = 57$) and significantly higher than the mean for cases in which noscapine was detected only in urine (0.013 mg/L; $n = 7$; $p = .001$). The free morphine concentration in blood was also significantly higher in blood positive noscapine cases (0.41 mg/L) compared to urine only positive cases (0.18 mg/L). Similarly, the mean FM/TM ratio was significantly higher in cases where noscapine was detected in blood (0.62) compared to those where it was only detected in urine (0.43; $p = .022$).

7.5.3.5 Morphine in 6AM positive cases

Statistical comparisons for FM and TM were also carried out between cases in which 6AM could be detected in blood and cases where the metabolite could only be detected in VH (Table 40). In cases with detectable 6AM in blood ($n = 22$), the mean FM/TM ratio was 0.55. In cases where 6AM was detected only in the VH ($n = 35$) the ratio was found to be significantly lower (mean: 0.38; $p = .003$). The FM concentration also differed in these

cases; in the presence of 6AM in blood the FM concentration in FB and VH was 0.30 and 0.23 mg/L respectively, whereas in cases where 6AM was detected only in VH the concentrations were significantly lower (0.17 and 0.14 mg/L respectively; $p < .05$). The FM concentration in VH was also significantly higher in blood positive 6AM cases ($p = .008$). The TM concentrations did not differ significantly in blood with detectable 6AM (0.59 mg/L) and in blood in which 6AM was not detected (0.52 mg/L; $p = .065$).

7.5.3.6 Blood alcohol and diazepam

Alcohol concentrations ranged from not detected to 298 mg/dL. Including all cases ($n = 70$) the mean concentration was 49 mg/L. When considering only cases in which alcohol was detected ($n = 30$) the range was 7 – 298 mg/dL and the distribution across the observed range had a slightly positive skew (Appendix 35). Of the cases where alcohol was present, 47% were rapid deaths ($n = 14$) and 13% were delayed ($n = 4$). Alcohol was detected in 50 and 25% of all rapid and delayed deaths respectively. In comparing blood alcohol concentrations (BAC) in rapid and delayed deaths, means were calculated with the inclusion of cases in which alcohol was not detected. The mean BAC measured in the rapid death group was 67 mg/dL, significantly higher than in the delayed death group (14 mg/dL; $p = .047$; Table 41). The difference in the mean distribution of the BAC in rapid and delayed deaths is illustrated by the box plot in Appendix 35. In contrast to previous reports (Ruttenber and Luke 1984; Steentoft et al. 1988; Ruttenber et al. 1990; Fugelstad et al. 2003; Darke et al. 2007) there was no significant inverse relationship between blood alcohol and morphine concentration in this study ($r = -.053$; $p > .05$; Appendix 36). The mean free morphine concentration in the presence of alcohol (0.18 mg/L) was not significantly different to cases in which alcohol was not detected (0.23 mg/L; $p = .286$).

In 30% of cases where diazepam was detected it was at a concentration < 0.16 mg/L. High diazepam concentrations (> 0.9 mg/L) were observed in 23% of cases (Appendix 37). In contrast with blood alcohol the mean blood diazepam concentration was higher in delayed deaths (0.75 mg/L) compared with rapid deaths (0.54 mg/L) although this difference was not significant ($p = .404$; Table 41). The box plot in Appendix 36 further illustrates the comparable distribution of diazepam concentrations in rapid and delayed deaths. No

correlation was observed between blood diazepam and free morphine concentration ($r_s = .060$; $p > .05$; Appendix 36).

Table 39 Summary data and mean comparisons of 6AM, free morphine, total morphine and the free to total morphine ratio obtained in cases where noscapine was detected in blood and where noscapine was only detected in urine

Matrix	Analyte		Noscapine detection matrix		Rapid-delayed group comparison		
			Blood (n = 10)	Urine only (n = 7)	<i>U</i>	<i>z</i> -score	Sig (<i>p</i>)
VH	6AM (mg/L)	Mean	0.057	0.013	3.50	-3.084	.001*
		Mdn	0.041	0.013			
		Range	0.020 – 0.13	0 – 0.028			
		SD	0.037	0.010			
Blood	6AM (mg/L)	Mean	0.003	0.001	21.00	-1.531	.071
		Mdn	0.002	0			
		Range	0 – 0.014	0 – 0.009			
		SD	0.005	0.003			
VH	FM (mg/L)	Mean	0.34	0.15	18.00	-1.659	.054
		Mdn	0.24	0.14			
		Range	0.12 – 1.1	0.029 – 0.34			
		SD	0.29	0.12			
Blood	FM (mg/L)	Mean	0.41	0.18	7.00	-2.734	.002*
		Mdn	0.38	0.22			
		Range	0.15 – 0.89	0.011 – 0.27			
		SD	0.21	0.095			
	TM (mg/L)	Mean	0.65	0.56	30.00	-.488	.335
		Mdn	0.56	0.54			
		Range	0.79 – 1.1	0.013 – 1.1			
		SD	0.26	0.34			
	FM/TM	Mean	0.62	0.43	14.00	-2.049	.022*
		Mdn	0.63	0.35			
		Range	0.34 – 0.78	0.21 – 0.85			
		SD	0.12	0.21			

Mdn: median; SD: standard deviation

* Significant Mann Whitney test result ($p < .05$ for a 1-tailed test) in the comparison of means for blood positive and VH only positive noscapine cases

Table 40 Summary data and group comparisons of free morphine, total morphine and the free to total morphine ratio obtained in cases where 6AM was detected in blood and where 6AM was only detected in vitreous

Matrix	Analyte		6AM detection matrix		Rapid-delayed group comparison		
			Blood (n = 22)	VH only (n = 35)	<i>U</i>	<i>z</i> -score	Sig (<i>p</i>)
VH	FM (mg/L)	Mean	0.23	0.14	238.00	-2.410	.008*
		Mdn	0.17	0.10			
		Range	0.028 – 0.57	0.028 – 1.1			
		SD	0.17	0.18			
Blood	FM (mg/L)	Mean	0.30	0.17	167.50	-3.566	.003*
		Mdn	0.29	0.16			
		Range	0.083 – 0.64	0.011 – 0.89			
		SD	0.14	0.15			
	TM (mg/L)	Mean	0.60	0.52	274.00	-1.529	.065
		Mdn	0.59	0.44			
		Range	0.11 – 1.1	0.013 – 1.2			
		SD	0.25	0.33			
	FM/TM	Mean	0.55	0.38	203.00	-2.749	.003*
		Mdn	0.55	0.33			
		Range	0.18 – 1.1	0.08 – 0.85			
		SD	0.23	0.21			

Mdn: median; SD: standard deviation

* Significant Mann Whitney test result ($p < .05$ for a 1-tailed test)

Table 41 Mean blood alcohol and diazepam concentrations grouped by mode of death with rapid versus delayed group comparisons

Analyte		Overall (n = 69)	Rapid (n = 28)	Delayed (n = 16)	Rapid-delayed group comparison		
					<i>U</i>	<i>z</i> -score	Sig (<i>p</i>)
Alcohol (mg/dL)	Mean	49	67	14	12.00	-1.700	.047*
	Mdn	0	27	0			
	Range	0 – 298	0 – 298	0 – 188			
	SD	76.5	87	47			
Diazepam (mg/L)	Mean	0.76	0.54	0.75	153.00	-.258	.404
	Mdn	0.38	0.31	0.36			
	Range	0 – 8.8	0 – 2.5	0 – 5.7			
	SD	1.4	0.64	1.4			

Mdn: median; SD: standard deviation

* Significant Mann-Whitney test result ($p < .05$ for a 1-tailed test)

7.6 Discussion

The majority of deaths investigated in this study were confirmed to be heroin related and several were suspected. Heroin-related deaths were classified as such based primarily on presence of 6AM, which was detected in at least one matrix in 60 cases. In two cases the use of heroin prior to death was confirmed by a witness. The presence of fresh injection sites and/or the presence of morphine in known heroin abusers were also considered to be indicative of recent heroin use. Thus in 68 of the 70 cases, heroin intake prior to death was assumed. In the remaining two cases, 30 and 69, death was attributed to a massive oral morphine overdose and a DHC overdose respectively. Only conjugated morphine was detected in blood in case 69 (0.27 mg/L) suggesting that if heroin had been used it was remote.

Compared with blood, the likelihood of detecting 6AM was increased substantially in VH. Of the 60 heroin exposures determined by the detection of 6AM, 93% were confirmed through the analysis of VH (n = 56), whereas only 37% were confirmed in blood (n = 22). The extended window of 6AM detection provided by the VH has been reported previously; in 36% of cases (n = 25) investigated by Wyman and Bultman (2004) and in 50% of cases (n = 12) investigated by Antonides et al. (2007), 6AM would not have been detected without the assay of VH. The concentrations of 6AM in VH are reported to consistently exceed those in blood (Pragst et al. 1999; Antonides et al. 2007) and this was also observed in the present study. In blood, 6AM could be detected at a concentration above the limit of quantification (LOQ: 0.005 mg/L) in only 9 cases, whereas in VH the metabolite could be quantified in 46 cases. It follows that the concentration of 6AM in the VH (mean: 0.031 mg/L) was significantly higher than in the blood (mean: 0.002 mg/L) with maximum concentrations of 0.15 and 0.019 mg/L measured respectively.

The relatively high concentration of 6AM observed in the VH may be attributed to 1) the high lipophilicity of heroin and 6AM allowing easy penetration of the blood-VH barrier; 2) subsequent *in situ* deacetylation of heroin to 6AM, which is known to occur rapidly at the pH of the VH (pH 7.5) and; 3) the lack of esterase activity in VH preventing the enzyme mediated conversion of 6AM to morphine. The significantly higher mean concentration of 6AM in VH measured in rapid deaths (0.048 mg/L; n = 26) compared with delayed death

(0.013 mg/L; $n = 10$; $p = .006$) reflects the greater elimination of 6AM from the VH during extended survival periods. 6AM was found to be highly correlated between FB and VH when considering all cases ($r_s = .604$; $p < .01$; $n = 57$). A correlation was also observed in rapid deaths ($r_s = .783$; $p < .01$; $n = 26$) whereas in delayed deaths no correlation was observed ($r_s = .156$; $p > .05$; $n = 10$). This is in contrast to the findings of a previous study (Scott and Oliver 1999), in which 6AM was not found to be correlated between VH and FB in 20 cases of heroin related death, including when only sudden deaths ($n = 17$) were taken into account (Table 42). Potential explanations for the observed difference may relate to 1) the present study examined a larger number of 6AM positive cases ($n = 57$) than Scott and Oliver ($n = 20$); 2) Scott and Oliver analysed cardiac blood, which is known to be subject to post-mortem redistribution, and; 3) unlike the present method, the method utilised by Scott and Oliver did not appear to have been validated and thus the validity of their results cannot be confirmed.

6AM is reported to only be detectable in blood for 2 – 3 hours following heroin intake (Rook et al. 2006a) and thus its presence in a blood sample is regarded as an indicator of short survival time following heroin intake (Cone et al. 1991; Goldberger et al. 1994; Burt et al. 2001). Of the 22 cases where 6AM was detected in the blood in the present study, 15 were known to be rapid deaths. In two delayed cases (11 and 45) 6AM was detected in blood at a concentration below the LOQ. In case 11, death occurred 3 – 3.5 hours following heroin injection and thus, according to the study criteria, death was on the verge of being rapid. In case 45 the victim smoked heroin just after at midnight and drank alcohol until 3am when a witness reported that he fell asleep. The person was not checked on again until six hours later when he was found dead. Based on the presence of 6AM in the blood, death may have occurred soon after the person fell asleep and thus closer to three hours following drug intake. Overall, the findings of this study further support the theory that the presence of 6AM in blood is a reliable indicator of rapid death. The absence of 6AM in blood however, is not necessarily indicative of the opposite. In 13 rapid death cases 6AM could only be detected in the VH.

The use of FM/TM concentration ratios as a means of evaluating the time of survival following heroin or morphine intake has been advocated in several studies (Garriott and

Sturner 1973; Reed 1979; Spiehler and Brown 1987; Staub et al. 1990; Cone et al. 1991; Mitchell et al. 1991; Goldberger et al. 1994; Burt et al. 2001) and may be of use in cases in which 6AM is not detectable in the blood. A high concentration of free morphine relative to that of the conjugated metabolites is thought to reflect insufficient time for metabolism and thus indicative of rapid death. FM/TM ratios between 0.51 and 0.76 have been associated with rapid death whereas mean ratios of 0.31 – 0.34 were associated with delayed death or the result for all cases (Spiehler and Brown 1987; Staub et al. 1990; Burt et al. 2000). Blood free and total morphine concentrations reported by these authors are summarised in Table 43 alongside the results obtained in blood in the present study. The findings of the present study are in agreement with these previous reports. Rapid deaths were characterised by a significantly higher FM/TM ratio in FB (mean: 0.55) compared to delayed deaths (mean: 0.26; $p < .001$). In assessing the FM/TM ratios in cases with detectable 6AM in blood ($n = 22$) and in cases where the metabolite was only detected in VH ($n = 35$), the mean FM/TM ratio in blood positive cases (mean: 0.55) was the same as the overall mean for rapid deaths. In the VH only positive cases the mean FM/TM ratio (0.38) was significantly lower than in the blood positive cases ($p = .003$). The ratio in VH only positive cases is somewhat higher than the overall mean for delayed deaths (0.26) and reflects the fact that almost half of all rapid deaths were in the VH only positive group ($n = 13$).

Whilst the presence of 6AM combined with a high FM/TM ratio appears to be a sound indicator of rapid death, the FM/TM ratio alone is not quite so reliable. In case 45, where 6AM was detected in blood and death was thought not to have occurred much more than three to four hours after heroin intake, the FM/TM ratio was very low (0.18). A low FM/TM ratio (0.21) was also observed in case 33, a rapid death case in which 6AM was quantifiable in blood. In the presence of 6AM in blood, a significant glucuronide concentration relative to free morphine could reflect a recent heroin exposure preceded by repeated heroin dosing. The morphine glucuronides (MG) have longer half-lives compared with morphine (Tollison et al. 2002), and the MG half-lives may be increased substantially in tolerant individuals (Gyr et al. 2000). Thus, in regular heroin users the glucuronides may accumulate (Tollison et al. 2002) relative to morphine resulting in a low FM/TM ratio

despite very recent exposure. Interpretation based solely on the FM/TM ratios in these two cases would have led to erroneous conclusions with respect to survival time.

Table 42 Opiate correlation data in blood and vitreous in the present study and in the previously published literature

Ref		N	Morphine	Codeine	6AM
[1]*	All cases	70	$r_s = .782^*$	$r_s = .672^*$	$r_s = .604^*$
	Rapid death	28	$r_s = .756^*$	$r_s = .482^*$	$r_s = .783^*$
	Delayed death	16	$r_s = .600^*$	$r_s = .695$	$r_s = .153$
[2]**	All cases	20	$R^2 = .697$		$R^2 = .006$
	Rapid death	17	$R^2 = .885$		$R^2 = .003$
[3]*	All cases	52	$r = .716^*$		
[4]*	All cases	40	$r = .26$		

[1] Present study; [2] Scott and Oliver, 1999; [3] Stephen et al. 2006; [4] Gerostamoulos and Drummer 1997

* Analytes analysed in femoral blood; ** site of blood collection not specified

r_s = Spearman's Rho correlation coefficient; r = Pearson's correlation coefficient;

R^2 = coefficient of determination; * Correlation is significant ($p < .05$)

Over-reliance on morphine to metabolite ratios in the assessment of survival time has been strongly advised against (Skopp et al. 1996; Karch 2009). In addition to survival time and metabolite accumulation in chronic users, multiple factors influence the concentrations of the parent compound and its conjugated metabolites in blood, even in the living. In a systematic review of 57 studies, Faura et al. (1998) reported huge variations in the MG to free morphine ratio measured in living patients. In particular, MG/FM ratios were higher in individuals with renal impairment, and routes of administration that avoided first pass metabolism, i.e. intravenous and intramuscular, resulted in lower metabolite production compared with oral administration. Individual/genetic differences, i.e. UDP-glucuronosyltransferase polymorphisms, are known to occur (Coffman et al. 1998; Tukey

and Strassburg 2000; Karch 2009) and may also account for some of the observed variation.

Table 43 Free and total morphine concentration data in blood and vitreous in the present study and in the previously published literature

Ref	MOD	N		Mean blood concentration (mg/L)		
				FM	TM	FM/TM ratio
[1]*	All cases	70	Mean	0.27	0.66	0.43
			Mdn	0.18	0.46	0.39
			SD	0.58	0.93	0.22
			Range	0-4.8	0.013-10	0-1.0
	Rapid (< 3h)	28	Mean	0.26	0.5	0.55
			Mdn	0.28	0.48	0.56
			SD	0.13	0.22	0.18
			Range	0.069-0.64	0.095-1.0	0.19-0.90
	Delayed (> 3h)	16	Mean	0.12	0.48	0.26
			Mdn	0.12	0.44	0.22
			SD	0.085	0.3	0.14
			Range	0-0.26	0.036-1.2	0-0.54
[2]**	All cases	52	Mean	0.71	1.09	0.69
			Mdn	0.60	0.88	0.73
			SD	0.52	0.78	0.22
			Range	0.05-2.05	0.05-3.9	0.2-1.0
	Rapid (< 3h)	44	Mean	0.78	1.09	0.76
			Mdn	0.65	0.88	0.75
			SD	0.52	0.78	0.16
			Range	0.05-2.05	0.05-3.9	0.5-1.0
	Delayed (> 3h)	8	Mean	0.34	1.10	0.31
			Mdn	0.33	0.95	0.33
			SD	0.26	0.83	0.08
			Range	0.05-0.5	0.2-2.3	0.4-0.4
[3]**	All cases	200	Mean	0.25	0.65	0.41
			Mdn	0.18	0.46	0.4
			SD	0.27	0.81	0.24
			Range	0-2.2	0.035-8	0-1.0
	Rapid (< 3h)	56	Mean	0.39	0.62	0.65
			Mdn	0.36	0.54	0.62
			SD	0.24	0.35	0.19
			Range	0.08-1.7	0.1-2.2	0.27-1.0

Table 43 Continued

Ref	MOD	N		Mean blood concentration (mg/L)		
				FM	TM	FM/TM ratio
[4]*	All cases	91	Mean	0.42	2.2	0.19
			SD	1.58	5	0.23
			Range	0-14.5	0.11-37	0-0.83
	Rapid [†]	23	Mean	0.26	0.93	0.35
			SD	0.21	0.68	0.25
			Range	0-0.87	0.31-3.0	0-0.83

[1] Present study; [2] Staub et al. 1990; [3] Spiehler and Brown 1987; [4] Burt et al. 2001

MOD: mode of death (rapid, delayed, all cases); N: number of cases; FM: Free morphine; TM: total morphine; FM/TM: free morphine to total morphine ratio

Mdn: median; SD: standard deviation; * Measurement in femoral blood; **site of blood collection not specified; [†] Rapid deaths characterised by presence of 6AM in femoral blood

The situation after death is even more complex as post-mortem effects can significantly alter the relative concentrations of morphine and its glucuronides. Owing to the relatively large volume of distribution (V_d) of morphine (3 – 5 L/kg) (Moffat et al. 2004), the drug distributes into tissues ante-mortem with less than 2% of a given dose circulating in blood (Karch 2009). It is thought that post-mortem release of free morphine from tissue to blood may easily double the morphine concentration in the latter (Skopp et al. 1996; Bogusz 1997). Since the morphine glucuronides have low volumes of distribution (≤ 0.15 L/kg) (Hunt et al. 1999) and thus predominantly distributed in plasma, the post-mortem elevation of free morphine in blood would invalidate the use of morphine to metabolite ratios in estimating survival time (Skopp et al. 1996; Karch 2009). Further, hydrolysis of morphine glucuronides back to free morphine as a result of bacterial enzymes occurs during the post-mortem interval and can alter the ratios significantly as a function of time (Moriya and Hashimoto 1997; Carroll et al. 2000; Skopp et al. 2001b). Given that the interpretation of FM/TM ratios measured in the early post-mortem period may be questionable, the reliable interpretation of free and total morphine concentrations in putrefying blood and tissues is impossible.

Free morphine concentrations in blood were influenced significantly by survival time with higher mean FM concentration measured in rapid (0.26 mg/L) compared with delayed deaths (0.12 mg/L; $p < .001$). An association between short survival time and higher free morphine concentrations in blood has been reported previously (Staub et al. 1990; Table 43). In subjects dying rapidly after heroin injection ($n = 44$) Staub et al. (1990) observed a mean FM concentration of 0.78 mg/L. In cases in which death occurred after a survival period ($n = 8$), the mean was much lower (0.34 mg/L). In the present study, the mean FM concentration measured in cases in which 6AM was detected in blood was 0.30 mg/L, slightly higher than that observed overall for rapid deaths and significantly higher than in cases where 6AM was only detected in the VH (0.17 mg/L; $p = .003$). The mean FM concentration in blood positive 6AM cases was comparable to that reported by Burt et al. (2001) (0.26 mg/L) in 23 heroin related deaths with detectable 6AM in blood.

In contrast, survival time did not significantly influence the concentration of FM in VH, although the mean was higher in rapid deaths (0.20 mg/L) compared with delayed deaths (0.15 mg/L; $p > .05$). The concentrations of morphine in VH are typically reported to be lower than corresponding FB concentrations (Pragst et al. 1994; Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007). Blood and VH opiate concentrations previously reported in the literature are summarised in Table 44. The findings of the present study, also summarised in Table 44, were in agreement with these previous reports. The concentration of morphine measured in FB (mean: 0.27 mg/L; mdn: 0.18 mg/L) was found to be significantly higher than in VH (mean: 0.18 mg/L; mdn: 0.12 mg/L; $n = 70$; $p = .001$). Morphine is lipophilic, although less so than heroin and 6AM, and its pK_a (8.0) would indicate that approximately 80% of the drug is in the non-ionised form at physiological pH (pH 7.4) and thus accessible for diffusion across the blood-vitreous barrier. The typically low morphine concentrations observed in the VH may be more heavily influenced by protein-binding, which for morphine is relatively high (20 – 35%) (Moffat et al. 2004). Since only free drug is able to leave the blood and enter the VH the movement of compounds into the VH will be determined by the protein-binding, with more highly bound drugs having smaller equilibrium concentrations in VH (Pragst et al. 1999; Lin et al. 1997).

Equilibrium between blood and VH is reported to be slower than between blood and other extracellular fluids (Levine and Jufer 2008). Thus, a lag in the distribution of free morphine into the VH would be expected. When rapid and delayed deaths were considered separately, this lag was evident; the VH/FB ratios measured in delayed deaths ranged between 0.23 and 5.2, whereas in rapid deaths the range was much smaller (0.31 – 1.7). The mean VH/FB ratio in delayed deaths (1.7; mdn: 0.98) was higher than in rapid deaths (0.75; mdn: 0.64), although the difference was not significant ($p > .05$). Similar findings were reported by Pragst et al. (1994), who examined 154 opioid related fatalities and found that in cases with a survival time greater than five hours, the VH/FB ratios were much higher compared with cases in which the survival time was short (Table 44).

The tendency for lower VH/FB ratios in rapid death may be linked particularly with insufficient time for complete distribution of the drug into the VH prior to death. In case 30, in which death was attributed oral morphine poisoning, the VH/FB ratio was very low (0.09); the concentration of morphine was 0.42 mg/L in the VH and 4.8 mg/L in the blood. Although survival time in this case was not known, the FM/TM ratio was relatively high (0.49) and the combined toxicological evidence is suggestive of rapid death, with the caveat that post-mortem diffusion of free morphine from gastric residues to blood (Pounder et al. 1996) could affect these ratios in a similar manner. High VH/FB ratios may be more indicative of an extended survival period allowing time for distribution and equilibration. Further, a very high VH/FB ratio may reflect the more rapid elimination of free morphine from blood compared with from VH. Glucuronidation does not occur in the VH so free morphine should be cleared more slowly from this fluid than it is from the blood. The result should be an increase in the VH/FB free morphine ratio as a function of survival time. In the present study however, substantial overlap in the VH/FB ratios was observed in the rapid and delayed death groups; in five of the 28 rapid death cases the VH/FB ratio exceeded 1.0, and in four of the 16 delayed cases the ratio was < 0.4 . Considering these findings, assessments of survival time based on this ratio alone would not be recommended.

A high VH morphine concentration relative to that in blood (VH/FB ratio > 1) could also result from repeated heroin/morphine administration and subsequent accumulation of morphine in this fluid. This occurrence could result in a high VH/FB ratio even in deaths

that occurred rapidly. In the majority of the cases studied here, heroin abuse was reported to be regular or chronic. In two cases of rapid death (case 1 and 56) where 6AM was detectable in blood and very high FM/TM ratios (0.90 and 0.69 respectively) were observed, the VH morphine concentration exceeded that in the blood with respective VH/FB ratios of 1.7 and 1.5. The relatively high VH concentration in these cases might be attributed to accumulation of morphine in the VH resulting from repeated heroin use in the hours/days preceding the final fatal dose.

Several factors may affect the VH/FB concentration ratio. Individual variability in the level of protein binding is known to exist (Karch 2009) and would influence the amount of free morphine entering the VH. Deconjugation of morphine metabolites and redistribution of morphine from tissues to blood during the post-mortem interval may also alter the VH/FB free morphine ratio significantly yet unpredictably. Very little is known about the behaviour of morphine in the VH after death. In a porcine model, concentrations of morphine in the VH were reported to increase within the first hour after death and to slowly decline thereafter (Crandall et al. 2006b). As a consequence of its isolated location within the eye, it is likely that the VH is less affected by post-mortem change compared with the blood, but since data in human subjects have not been reported the truth of this statement is unclear. Despite large variation in the relative concentration of morphine in FB and VH, which was generally a function of survival time, an excellent correlation was observed between the two matrices in all groups. When including all cases, a Spearman's rho coefficient (r_s) of .782 was observed ($p < .01$). The correlation between morphine in blood and VH has been previously reported to be dependent on survival time (Scott and Oliver 1999). These authors observed a higher correlation when only sudden deaths were taken into account ($r = .885$; $n = 17$) compared to that observed when all cases were included ($p = .697$; $n = 20$; Table 42). In the present study the correlation between morphine in FB and VH was also influenced by survival time, with a higher correlation observed in rapid deaths ($r_s = .756$; $p < .01$) compared to delayed deaths ($r_s = .600$; $p < .01$). The lower correlation in delayed deaths reflects the wider range in the VH/FB ratios observed in this group.

Table 44 Opiate summary data in blood and vitreous obtained in the present study and in the published literature

Ref	MOD	N		Blood concentration (mg/L)				Vitreous humour concentration (mg/L)				VH/FB ratio		
				FM	6AM	Codeine	C/FM	FM	6AM	Codeine	C/FM	FM	6AM	COD
[1]*	All cases	70	Mean	0.27	0.002 (n=58)	0.031	0.16	0.18	0.031 (n=57)	0.041	0.35	0.99	12	1.5
			Mdn	0.18	0.002	0.026	0.14	0.12	0.02	0.028	0.28	0.76	8.3	1.2
			SD	0.58	0.004	0.035	0.11	0.18	0.035	0.038	0.28	0.93	13	1.1
			Range	0-4.8	0-0.019	0-0.25	0-0.63	0.004-1.1	0-0.15	0.002-0.17	0.01-1.5	0.22-5.2	0-55	0.09-6.1
	Rapid < 3h	28	Mean	0.26	0.004 (n=26)	0.031	0.13	0.2	0.048 (n=26)	0.043	0.25	0.75	15	1.4
			Mdn	0.28	0.002	0.028	0.11	0.13	0.033	0.031	0.23	0.64	10	1.2
			SD	0.13	0.005	0.021	0.049	0.14	0.043	0.033	0.12	0.34	14	0.74
			Range	0.069-0.64	0-0.019	0.012-0.13	0.07-0.24	0.046-0.57	0.002-0.15	0.01-0.17	0.1-0.58	0.31-1.7	3.0-55	0.32-3.5
	Delayed > 3h	16	Mean	0.12	0.001 (n=10)	0.034	0.17	0.15	0.013 (n=10)	0.032	0.37	1.7	5	1.6
			Mdn	0.12	0	0.019	0.14	0.14	0.009	0.02	0.27	0.98	5	1
			SD	0.085	0.002	0.06	0.14	0.13	0.011	0.028	0.31	1.7	2.8	1.5
			Range	0-0.26	0-0.004	0-0.25	0-0.58	0.004-0.41	0.001-0.032	0.002-0.086	0.01-1.0	0.23-5.2	3.0-7.0	0.09-6.1
[2]*	All cases	25	Mean	0.129	0.012	0.008	0.057	0.041	0.027	0.008	0.173	0.36	11.3	0.83
			Mdn	0.11	0.005	0.01	0.0625	0.03	0.014	0.01	0.2	0.33	9	1
			SD	0.11	0.026	0.008	0.055	0.038	0.035	0.01	0.155	0.22	9.4	0.42
			Range	0.01-0.57	0.001-0.09	0-0.04	0-0.2	0-0.2	0.002-0.17	0-0.05	0-0.5	0-1.0	1.7-2.8	0-1.3
[3]**	All cases	154	Mdn	0.56				0.28				0.65		
	Rapid < 2h	34	Mdn	0.58				0.26				0.57		
	Mod. 2-5h	14	Mdn	0.56				0.24				0.65		
	Delayed > 5h	12	Mdn	0.1				0.14				3.3		
[4]**	All cases	29	Mean	0.73				0.14	0.013			0.43		
			Mdn	0.43				0.11	0.007			0.27		
			SD	1.1				0.13	0.018			0.57		
			Range	0.03-5.8				0-0.26	0-0.08			0-3.0		

Table 44 continued

				Mean FB concentration (mg/L)				Mean VH concentration (mg/L)				VH/FB ratio		
Ref	MOD			FM	6AM	Codeine	C/FM	FM	6AM	Codeine	C/FM	FM	6AM	Codeine
[5]**	All cases	20	Mean	0.66	0.096			0.51	0.24			0.89	3.7	
			Mdn	0.47	0.045			0.37	0.04			0.85	0.47	
			SD	0.55	0.14			0.46	0.44			0.45	6.0	
			Range	0.05-2.1	0-0.51			0.07-1.7	0-1.7			0.1-1.9	0-17	
	Rapid < 3h	17	Mean	0.65	0.074			0.55	0.25			0.94	4.6	
			Mdn	0.45	0.01			0.42	0.05			0.88	0.69	
			SD	0.56	0.13			0.48	0.47			0.44	6.7	
			Range	0.05-2.1	0-0.51			0.07-1.7	0-1.7			0.1-1.9	0-17	

[1] Present study; [2] Wyman and Bultman 2004; [3] Pragst et al. 1994; [4] Pragst et al. 1999; [5] Scott and Oliver 1999.

MOD: Mode of death (rapid, delayed, all cases); N: number of cases; FM: free morphine; C/FM: codeine to free morphine ratio

* Analytes measured in femoral blood; ** site of blood collection not specified

Mdn: median; SD: standard deviation

Note: Pragst et al. 1994 [3] only reported median data so mean, SD and range could not be presented

In contrast to free morphine, the concentration of codeine in VH (mean: 0.041 mg/L) was found to be significantly higher than in FB (mean: 0.031 mg/L; $p < .001$). This finding is clearly illustrated in Figure 26. Survival time significantly influenced the concentration of codeine in FB with higher concentrations measured in rapid (mean: 0.031 mg/L; mdn: 0.028 mg/L) versus delayed deaths (mean: 0.034 mg/L; mdn: 0.019 mg/L). Survival time did not significantly influence the concentration of codeine in the VH. When considering all cases, the codeine/free morphine (C/FM) concentration ratio was found to be significantly higher in VH (mean: 0.34; range: 0.01 – 1.50) compared with FB (0.16; range: 0 – 0.63; $p < .001$). Significant differences between VH and FB C/FM ratios were also observed in rapid (VH mean: 0.25; FB mean: 0.13; $p < .001$) and delayed (VH mean: 0.37; FB mean: 0.17; $p = .009$) deaths. The observation of higher C/FM ratios in VH compared with blood is in agreement with the findings of Wyman and Bultman (2004) (Table 44). In 20 heroin related deaths they reported a mean C/FM ratio of 0.059 (0.03 – 0.10) in FB and 0.20 (0.07 – 0.35) in VH ($n = 18$). The higher C/FM ratios in VH as compared with blood might be attributed to the difference in the plasma-protein binding of the two drugs. The protein binding of codeine is typically reported to be lower than that of morphine with a range of 7 – 25% (Moffat et al. 2004). Consequently, more free codeine would be available to cross the blood-vitreous barrier compared with morphine. The lower protein binding of codeine would also explain why, in contrast to free morphine, the codeine concentration was frequently higher in VH compared to FB.

It has been suggested that in the absence of 6AM the relative concentrations of morphine and codeine can be utilised to distinguish between morphine present as a result of heroin, and morphine metabolised from codeine following medicinal use of the latter (Lin et al. 1997; Gill and Graham 2002; Wyman and Bultman 2004). It has been demonstrated that finding a codeine to morphine ratio > 1.0 in blood provides evidence of medicinal codeine use (Nakamura et al. 1976). A ratio < 1.0 , either in blood or VH, is suggested to be indicative of heroin use (Lin et al. 1997; Wyman and Bultman 2004). In only one case in the current study was the use of medicinal codeine evident. In case 69, a codeine concentration of 0.25 mg/L was measured in blood. Total morphine measured 0.27 mg/L with no free morphine detected. In all of the heroin related deaths investigated in this study, the C/FM ratios were in the range reported for heroin exposures.

Caution must be applied when using C/FM ratios to discern heroin exposures. In one case, in which death was attributed to oral morphine poisoning with prescription Oramorph and morphine sulphate tablets, C/FM ratios of 0.01 and 0.14 were observed in FB and VH respectively. These ratios are consistent with heroin exposure yet this individual was 61 years old, had no history of heroin abuse and there was no evidence of recent or old injection sites and no indicators of heroin use at the scene of death. It would appear highly unlikely that this individual had taken heroin prior to death. When interpreting C/FM ratios other potential sources of codeine must be considered. A low C/FM ratio could also result from contamination of commercial morphine preparations (Baselt 2004), from remote use of over-the-counter or prescription codeine, or as a result of poppy seed ingestion. The latter scenario may be distinguished by the analysis of thebaine, a naturally occurring opiate introduced into the body along with morphine and codeine following poppy seed ingestion (Meadway et al. 1998). However, the absence of thebaine does not rule out poppy seeds as the source of morphine and codeine. Given the potential and not uncommon sources of codeine, its use as a marker of heroin exposure in the absence of more specific markers, i.e. 6AM, is not recommended.

More reliable indicators of heroin use include the detection of noscapine, papaverine and their respective metabolites. These compounds are constituents of the opium poppy and both the parent compounds and their respective metabolites are reported to provide highly specific markers in the determination of illicit heroin use (Bogusz et al. 2001; Paterson et al. 2005). Although noscapine is used in antitusive preparations in Europe (McLachlan-Troup et al. 2001), it is not licensed for use in the UK. Papaverine is available in the UK as an analgesic and for the treatment of impotence and cardiovascular disease, although it is rarely prescribed (Paterson et al. 2005). Like codeine, noscapine and papaverine metabolites have been detected in the urine of individuals consuming large amounts of poppy seeds (McLachlan-Troup et al. 2001; Paterson et al. 2005). Thus, a degree of caution must be employed when interpreting heroin exposures based solely on the detection of these compounds. In all of the present cases where noscapine was detected ($n = 17$), 6AM was also detected thus confirming heroin exposure. In one noscapine positive case, papaverine was also detected.

An association between rapid death and the detection of noscapine in blood was observed in this study. Noscapine was detected in 17 cases either in blood ($n = 10$) or urine ($n = 7$). Of the 17 cases where noscapine was detected, death was known to have been rapid in ten and delayed in two. Of the ten rapid deaths noscapine could be detected in the blood in eight. In the two delayed death cases noscapine was only detected in urine. In cases in which noscapine was detected in the blood ($n = 10$) the mean FM/TM ratio was 0.62 compared with 0.43 in the urine only positive cases. The mean blood concentration of FM was 0.41 mg/L (0.15 – 0.89 mg/L) in blood positive noscapine cases. This was found to be significantly higher than that measured in urine only noscapine positive cases (mean: 0.18; range: 0.011 – 0.27 mg/L; $p = .002$) and 1.6 times higher than the mean FM concentration measured in all rapid deaths (0.26 mg/L). A significant difference between blood positive and urine only positive noscapine cases was also observed for 6AM in VH, with significantly higher concentrations observed in the blood positive noscapine cases (0.057 mg/L) compared to urine only positive cases (0.013 mg/L; $p = .001$).

The detection window of noscapine in blood has not been well characterised although its plasma half-life is reported to be longer than that of 6AM (1.5 – 4 hours) (Moffat et al. 2004). The longer half-life of noscapine compared with 6AM was evidenced in the present study by its more frequent detection in blood; of the ten cases in which noscapine was detected in the blood 6AM was detectable in only seven. Owing to its extensive metabolism in humans the detection of noscapine in the urine of living individuals is rare, and the detection of its primary metabolites, cotarnine and meconine, is more commonly reported (McLachlan-Troup et al. 2001). The detection of noscapine in post-mortem blood would therefore imply a very recent heroin exposure and the results of this study support this. Taylor and Elliot (2009) have also reported that in cases where rapid death following heroin injection was suspected, opium poppy constituents were commonly detected in the blood.

Although the findings of this study clearly indicate an association between noscapine detection in blood and rapid death, the absence of noscapine in a blood or urine sample would have no meaning in the assessment of survival time. The presence of noscapine is more commonly associated with the use of ammonia to isolate morphine from opium (common in Western Asia), rather than the method that utilises lime (common in South-east

Asia) (Bogusz et al. 2001). The presence of noscapine in illicit heroin depends therefore on the manufacturing process, and the frequency of its detection may vary over time and location, as found by Strang et al. (1997b). Thus, whilst the presence of noscapine in blood may be particularly useful as an indicator of rapid death following heroin intake, the fact that its detection also depends in the source of heroin limits its overall usefulness in the interpretation of heroin related deaths.

Toxicological indicators of survival time can aid substantially in assessing cause of death in drug-related fatalities. In many instances, relevant scene or witness information pertaining to survival time is not available and the toxicological findings are the only resource for interpretation. In the absence of trauma, underlying pathology or illness, death occurring rapidly following heroin intake is suggestive of a causal link. In contrast, the concentration of free morphine measured in a biological sample, namely blood, may be less helpful in determining the cause of death. A major impediment to the interpretation of heroin-related deaths is their frequent association with low blood morphine concentrations, often comparable to or lower than those observed in living heroin users and in cases of death where morphine was an incidental finding (Monforte 1977; Kintz and Mangin 1995; Zador et al. 1996). In agreement with these previous reports, the concentrations of morphine measured in this study were generally quite low. Blood FM concentrations of less than 0.3 mg/L were measured in 79% of all cases (Figure 28a) with an overall median of 0.18 mg/L. Only 5% of cases had morphine concentrations higher than 0.5 mg/L with the highest blood morphine concentration (4.8 mg/L) not associated with heroin but instead the result of an oral morphine overdose.

The typically low morphine concentrations observed in this study can, in part, be attributed to survival time, with the lowest free morphine concentrations (< 0.1 mg/L; $n = 20$) often associated with delayed deaths. In only three of these cases was death reported to be rapid. Death was known to be delayed in seven and the toxicological data in many of the remaining cases was also indicative of delayed death, i.e. 6AM was detectable only in the VH in seven cases and was below the LOQ (< 0.005 mg/L) in three of these, the FM/TM ratios were low in five cases (≤ 0.37 ; not analysed in two) and VH/FB ratios were > 1.0 in four cases. In addition to long survival periods, low blood morphine concentrations in cases

of heroin overdose have been attributed to periods of abstinence resulting in loss of tolerance (Tagliaro et al. 1998; Darke et al. 2002) and/or the concomitant use of other drugs (Monforte 1977; Darke et al. 1996; Zador et al. 1996; McGregor et al. 1998; Fugelstad et al. 2002; Coffin et al. 2003; Darke et al. 2007). Unfortunately information pertaining to drug abstinence prior to death was not available in this study. Polydrug use, on the other hand, was found to be highly prevalent in these cases.

Polydrug use has been documented as a key risk factor in overdose and overdose mortality and there is considerable evidence that many instances of opioid overdose are due to the combined effects of opioids with other drugs, particularly other CNS depressants, such as alcohol and benzodiazepines (Monforte 1977; Darke et al. 1996; Zador et al. 1996; McGregor et al. 1998; Coffin et al. 2003; Darke et al. 2007). In support of a polydrug mechanism is the frequently documented finding that cases in which morphine is the only drug detected at autopsy represent only a minority of heroin fatalities (Richards et al. 1976; Monforte 1977; Steentoft et al. 1988; Goldberger et al. 1994; Zador et al. 1996; Darke et al. 2000; Oliver and Keen 2003; Darke et al. 2010). In the present study morphine was detected in the absence of another substance (excluding 6AM and codeine) in only one case. The most frequently detected other substances were alcohol and diazepam, which were detected in 43 and 87% of cases respectively. In 33% of cases both alcohol and diazepam were present in combination with morphine. These drugs by themselves are relatively weak respiratory depressants but when combined with a potent respiratory depressant, such as morphine, are thought to augment the opioid effect on respiration (Darke et al. 1996; White and Irvine 1999).

Importantly, an inverse relationship between blood alcohol and morphine concentration has been consistently reported in heroin/morphine fatalities suggesting that alcohol use decreases the amount of heroin required to induce a fatal overdose (Ruttenber and Luke 1984; Steentoft et al. 1988; Ruttenber et al. 1990; Fugelstad et al. 2002; Darke et al. 2007). Zador et al. (1996) reported that the mean blood morphine concentration in alcohol positive cases (0.17 mg/L) was significantly lower compared to cases in which only morphine was detected (0.34 mg/L). In the present study the mean concentration of morphine in alcohol positive cases (0.18 mg/L; n = 30) was not significantly lower than that observed in cases

where alcohol was not present (0.23 mg/L; $n = 40$; $p = 0.29$) and although a negative relationship was observed between blood alcohol and morphine concentration ($r_s = - .053$; Appendix 36), this was also not significant ($p > .05$). However, the detection of alcohol was associated more frequently with rapid deaths (47%; $n = 14$) than it was with delayed deaths (13%; $n = 4$) and the mean blood alcohol concentration was significantly higher in rapid (67 mg/dL; mdn: 27 mg/dL) compared with delayed deaths (15 mg/L; mdn: 0 mg/dL; $p = .047$). It has been reported previously that the risk of heroin overdose is increased with increasing alcohol concentration (Ruttenber and Luke 1984; Fugelstad et al. 2002). Ruttenber and Luke (1984) determined that alcohol in excess of 100 mg/dL increased the probability of a heroin user experiencing a fatal intoxication by a factor of 22. In 64% of rapid deaths where alcohol was detected in the present study ($n = 9$) the measured concentration was ≥ 100 mg/dL. These findings support an increased risk associated with higher amounts of alcohol ingestion and further imply that following alcohol ingestion heroin overdose deaths might occur more suddenly.

The frequency of benzodiazepine (diazepam) detection in heroin/morphine involved deaths (87%; $n = 60$) was much higher in this study than has been previously reported. Studies investigating the frequency of detection of other drugs in heroin related deaths have reported benzodiazepines to be present in 9 to 40% of cases ($n = 23 - 959$) (Richards et al. 1976; Monforte 1977; Goldberger et al. 1994; Zador et al. 1996; Fugelstad et al. 2002; Darke et al. 2007; Darke et al. 2010). Although an increased risk of heroin overdose in the presence of benzodiazepines has been proposed, no significant relationship between the concentrations of morphine and benzodiazepines in blood has been found and the frequency of benzodiazepine detection in cases of heroin fatalities compare well with living heroin users (Zador et al. 1996; Darke et al. 1997; Fugelstad et al. 2002). In accordance with previous reports, no correlation was observed between blood morphine and diazepam concentration. Further, the detection of diazepam was not associated with rapid death. Diazepam was detected in 83% of delayed deaths ($n = 15$) and in 64% of rapid deaths ($n = 18$) and the mean concentration of diazepam in delayed deaths (0.80 mg/L) was actually higher than in rapid deaths (0.54 mg/L) although the median concentrations were more comparable (0.36 mg/L and 0.31 mg/L respectively). In one study (Fugelstad et al. 2002), the presence of flunitrazepam was associated with higher levels of morphine and 6AM in

blood. These authors proposed the possibility of a mechanism of interaction different to that involved with concomitant alcohol consumption. In the present study, the number of cases in which diazepam was not detected was insufficient for such a comparison to be made.

The high frequency of diazepam detection observed in the present study may simply be a reflection of the pattern of drug use in the population studied rather than an increased risk of overdose associated with concomitant heroin and diazepam consumption. The high prevalence of benzodiazepine use in Scotland has been demonstrated in a study of drug impaired Scottish drivers arrested in 1996 – 2000, 2003 and 2008 (Officer 2009). In 1996 – 2000 benzodiazepines were detected in 39% of cases (n = 102), whereas in 2003 and 2008 at least one drug of this group was detected in 85% (n = 24) and 83% (n = 295) of cases respectively. An earlier paper published by Seymore and Oliver (2000) discussed the role of alcohol and drugs in impaired drivers from the Strathclyde region over a five year period and at least one drug of the benzodiazepine group was detected in 82% of cases.

The relative contribution of diazepam to the cause of death in the cases investigated here is difficult to determine because an individual's level of tolerance to the drug is unknown. Toxic effects produced by diazepam alone are reported to occur at blood concentrations greater than 1.5 mg/L and fatalities, although rare, may occur at concentrations greater than 5 mg/L (Moffat et al. 2004). However, benzodiazepine concentrations > 0.9 mg/L have been reported to be relevant in heroin overdose deaths (Guitierrez-Cebollada et al. 1994). In the present study diazepam concentrations exceeding 0.9 mg/L were observed in 23% cases (n = 14). In each of these cases, with the exception of case 43 where death was a result of hanging, diazepam was listed as a contributory factor in cause of death.

In a large proportion of the cases studied here, the concentrations of diazepam could be considered therapeutic. The therapeutic concentration of diazepam in plasma has been reported to be between 0.1 – 1 mg/L with concentrations at the higher end of the range indicative of frequent use and increased tolerance (Moffat et al. 2004). In 77% of all cases where diazepam was detected in the present study (n = 60), the concentration was less than 0.9 mg/L (n = 46; Appendix 37). Oliver and Keen (2002) also reported that in a high percentage of opioid involved deaths, diazepam was present in largely therapeutic

concentrations and suggested that benzodiazepines, at least in lower concentrations, are a feature rather than a risk factor *per se* in heroin fatalities. These authors noted the importance of this distinction since benzodiazepines, unlike alcohol, may have a role to play in the treatment of drug misuse.

The most important risk factor for fatal overdose in the present study appears to relate to the route of heroin administration. Heroin was reported to have been injected intravenously in 42 cases. This constitutes 63% of all cases where heroin use prior to death was confirmed by 6AM detection or witness statements, or suspected based on knowledge of the individuals drug history and no current morphine prescription (n = 67). Of the 28 deaths classified as rapid in this study, 23 occurred following intravenous (IV) injection of heroin. Intravenous injection causes a rapid increase in brain levels of the drug increasing the likelihood of overdose via this route of administration (Frischer et al. 1997; Karch 2002; Kaye and Darke 2004). A British study found that 58% of 212 injecting heroin users had overdosed at some stage in their lives, with 30% having experienced an overdose in the previous year (Bennett and Higgins 1999). In another British study, Oliver and Keen (2002) reported that 80% of heroin deaths (n = 94) involved probable injecting. The high percentage of deaths attributed to IV heroin in this study may, therefore, be a reflection of the increased risk of overdose associated with this route of administration.

Confirmed smoking cases (n = 14) were less frequent compared with IV injection. In 11 cases the route of heroin administration was undetermined owing to the absence of fresh injection sites and witness statements. Smoking may have been the predominant route of administration in these undetermined cases although intranasal and oral administration is also a possibility. If the assumption was made that all these cases involved smoking heroin, the percentage of deaths from smoking would equate to 37% of the total, just over half that observed following the injection of the drug. The concentrations of heroin and its active metabolites build up more slowly when the drug is inhaled leading to increased control and less risk of overdose compared to the injection of a bolus dose (Klous et al. 2005). Further, the onset of intoxication will lead to respiratory depression, which in turn automatically leads to a reduction of heroin intake when smoking which may prevent a serious overdose.

The results of the present study indicate that death as a result of smoking heroin is less common than that resulting from injection of the drug.

7.7 Conclusion

The blood morphine concentrations measured in heroin related deaths depend on multiple factors. Whilst a very high morphine concentration may be consistent with the consequences of heroin toxicity, notwithstanding the issue of tolerance, the interpretation of low concentrations requires the consideration of more factors. In addition to loss of tolerance and drug-drug interactions, a low blood morphine concentration may simply be a reflection of an extended period between drug intake and death, which may be inferred from the relative amount of free morphine to conjugated morphine, with the caveat that these ratios may be altered during the post-mortem interval. Indicators of rapid death may be somewhat more reliable, particularly the blood detection of rapidly metabolised heroin specific compounds, i.e. 6AM and noscapine. A further adjunct to interpretation is provided by the assay of VH with its principal value being the extended period of detection it provides for 6AM. Although morphine appears to be well correlated between FB and VH, the relative concentrations in the two matrices are highly dependent on survival time and may be further influenced by accumulation of the drug in chronic users. Thus, in the absence of a blood sample, the blood concentration should not be inferred from that measured in the VH. Assessments based on the combined toxicological information obtained in FB and VH should greatly assist in the interpretation of heroin-related deaths, with additional information, i.e. scene information, witness statements, and knowledge of the individuals medical/drug history, increasing the likelihood of reaching an accurate determination of the mode and cause of death.

Chapter 8.0 Thesis discussion

Knowledge of the distribution of drugs within different body tissues and blood and the effect of post-mortem stability on this distribution is vital, not only for selecting appropriate specimens for toxicological examination, but also so that interpretation based on drug measurements made in these tissues can provide a reasonable level of reliability. This study was designed to investigate such relationships and to elucidate the interpretive value of thigh muscle and vitreous humour in opiate and cocaine related deaths. The difficulty associated with the interpretation of post-mortem blood drug concentrations has been reiterated throughout this thesis. In the first instance blood drug concentrations may not correlate with toxicity in a living individual (Ferner 2008) so the fact that concentrations in post-mortem blood do not necessarily reflect the situation at the time of death makes interpretation of concentrations measured in blood extremely difficult. Notwithstanding the post-mortem phenomena of site- and time-dependency, blood is the sample of choice for assessing pharmacological effects. However, owing to the persistence of cocaine metabolising enzymes after death, blood is of little interpretive value for cocaine determinations and in some instances blood may not be available at autopsy. It was proposed at the beginning of this thesis that tissues that are less affected by post-mortem change and redistribution might have the potential to provide more reliable specimens than blood for post-mortem toxicological determinations by virtue of increased drug stability. For drugs broken down by hydrolytic esterases, i.e. cocaine and 6AM, muscle tissue and vitreous humour should provide an extended window of detection owing to their lack of esterase activity. The utility of VH in this respect has been previously reported both for 6AM and cocaine (Pragst et al. 1999; Mackey-Bojack et al. 2000; Wyman and Bultman 2004; Duer et al. 2006; Antonides et al. 2007) and the findings of the present study were in accordance with these previous reports.

A single study has demonstrated the increased stability of cocaine in muscle compared with blood (Moriya and Hashimoto 1996). In the present study the unpreserved stability of cocaine was increased substantially in deer muscle compared with both horse blood and sheep VH (Chapter 4). Toxicological specimens in which drugs may be detected for longer than in blood are certainly advantageous in post-mortem drug determinations but the

interpretive value of such tissues relates more specifically to whether or not drug concentrations measured at the time of death correlate with that in blood. Great inter- and intra-muscular variation in the concentration of some basic drugs has been observed (Williams and Pounder 1997; Langford et al. 1998) and as a consequence the relationship between blood and muscle drug concentrations has barely been addressed. Although more data is available on the VH concentration of opiates (e.g. Pragst et al. 1994; Wyman and Bultman 2004) and cocaine (e.g. Mackey-Bojack et al. 2000; Duer et al. 2006), information on the relationship between VH and FB is too often based on small sample numbers or individual cases, particularly for opiates. The present study has provided valuable information on the relationship between FB and VH opiate concentrations in a large sample number and has highlighted the utility of these tissues in the assessment of survival time in heroin-related fatalities. Further, this study has provided the first report on the within muscle distribution of cocaine and has indicated that muscle tissue could have interpretive value in the post-mortem determination of this drug and its metabolites.

This research has provided valuable data on the stability of cocaine and 6AM in horse blood, a matrix that is used extensively in the UK for the preparation of calibration and check standards. The findings have highlighted the importance of sodium fluoride addition and frozen storage to ensure the stability of cocaine and 6AM in this matrix. These findings are highly significant since some laboratories store control standards in unpreserved horse blood for up to 6 months prior to use (pers. com. Gail Cooper 2011).

The following sections will summarise the pertinent study findings for cocaine and opiates and their interpretation in relation to previously published research. The study limitations and recommendations for future work will be discussed thereafter (Section 8.3).

8.1 The tissue distribution of cocaine

One of the major findings of this study was that cocaine and its metabolites (BZE and COET) were evenly distributed throughout the thigh muscle (ThM; *rectus femoris*) (Chapter 6). These findings are highly significant as they diverge from those of Williams and Pounder (1997) who reported great variation in the concentration of some other basic drugs, predominantly tricyclic antidepressants and benzodiazepines, throughout the thigh

muscle bulk. These authors noted that although drugs with the highest V_d tended to show the greatest variation, the variation of drugs with a low V_d was generally too great for the muscle to be of interpretive value in drug deaths. In another study investigating the same drug groups, substantial differences in drug concentration were also observed between several different muscles (Langford et al. 1998). As a consequence of these findings muscle is currently considered to be of use only for qualitative drug determinations. The findings of the present study suggest otherwise, at least for cocaine and its metabolites. The reason why the distribution observed in this study differs so greatly from that observed in the previous studies may relate to fact that only acute overdose deaths were investigated in the latter and so the variation these authors observed could be attributed to incomplete distribution associated with short survival times. In the majority of the cases analysed in the present study the circumstances of death implied a delay between cocaine intake and death. Further, death was a result of trauma in all but one case where the cause of death was unknown.

It has been suggested by Garriott (1991) that the relative concentration of cocaine in thigh muscle and blood was influenced by survival time. These authors observed a very low muscle to blood ratio (0.1) in two cases of acute cocaine death and suggested this to reflect insufficient time for drug distribution and tissue equilibration. It should be noted that the data provided by Garriott (1991) is somewhat limited because blood was sampled from the heart. The authors did not discuss the potential contribution of post-mortem elevation of drug concentration in aortic blood to the observed low ratios. Further, the analytical methods did not appear to have been validated which brings into question the reliability of the reported data. In the present study all cases were characterised by very high relative concentrations of cocaine in muscle with ThM/FB ratios between 2 and 4. The contribution of post-mortem redistribution and hydrolysis in blood was not considered to be significant since the samples were all very fresh; the time between death and sample collection ranged between 8 and 20 hours and in almost all cases autopsies were carried out within 10 – 15 hours of death, remarkably quick compared to the UK where an autopsy may not be carried out for several days following receipt of the body. Thus the relationship observed between blood and muscle should be fairly close to that at the time of death.

Whilst the high ThM to blood ratios observed in the present cases is indicative of substantial ante-mortem breakdown of cocaine in blood, in one case this observation did not appear to be the result of an extended period between drug intake and death. In the single case where cause of death was unknown, death was suspected to be an acute event following drug intake (case 7) yet the thigh muscle to blood ratio was also high (2.3). A shorter survival time in this case, at least compared with the other cases, was supported by the fact that this was the only case in which the concentration of BZE in the blood exceeded that in the muscle. Further, the concentration of cocaine in this case was much higher than that of BZE, both in ThM and VH (cocaine/BZE ratio of 2.7 and 2.2 respectively), whereas for all other cases the maximum cocaine/BZE ratio was 0.32 in TM and 0.82 in VH, indicative of substantial ante-mortem breakdown of cocaine in these tissues.

It can be concluded that a relatively high concentration of cocaine in muscle compared with blood may not necessarily be an indicator of prolonged survival time. Such an observation might also reflect repeated use of the drug prior to death leading to accumulation of cocaine in muscle. Accumulation of cocaine in deep body tissues has been previously suggested (Cone and Weddington 1989; Burke et al. 1990). In two cases involving the chronic therapeutic use of temazepam, Langford and Pounder (1998) reported comparable concentrations in several peripheral muscles, whereas there was great inter-muscular variation in most other cases. The authors suggested this finding to be indicative of tissue equilibration in chronic users. Thus, it can be postulated that both the survival period as well the pattern of cocaine use in the hours or days prior to death will likely affect the within-muscle distribution of cocaine and that in the present cases both factors may have contributed to the uniform distribution that was observed.

Because muscle lacks esterase activity and because the passage of BZE into the muscle is limited by its pharmacokinetic properties, it may be reasonable to postulate that the majority of the BZE detected in muscle is a result of *in situ* chemical hydrolysis, which would be expected to occur given the *in vivo* pH of skeletal muscle (~pH 7.0). In this respect the interpretive value of muscle may have some parallel with brain, which at present is thought to be the most reliable tissue for the interpretation of cocaine (Spiehler and Reed 1985; Stephens et al. 2004; Bertol et al. 2008). Since all the BZE present in brain

is formed *in situ* then a BZE concentration that is relatively low compared with that of cocaine is thought to reflect insufficient time for substantial cocaine breakdown thus indicating a short survival period. A high brain concentration of BZE with relatively low (or absent) cocaine concentration is thought to indicate substantial ante-mortem breakdown, and thus prolonged survival time, and/or accumulation resulting from repeated use.

It was postulated that the cocaine to BZE ratio observed in case 7 implied a shorter survival period compared with other cases because the ratio in ThM (2.7) was substantially higher compared to all other cases where the maximum cocaine/BZE ratio in ThM was 0.32. It is known that such inferences cannot be made in blood because 1) cocaine's rapid metabolism continues in blood after death, and; 2) the V_d of cocaine (1.9 – 2.4 L/kg) (Hart et al. 2000) is higher than the V_d of BZE (0.7 L/kg) (Ambre et al. 1991) and thus most of the cocaine will reside in the tissue and most of the BZE in the blood. These factors combined invalidate the use of cocaine/BZE ratios in blood in approximating survival time. Although the cocaine/BZE ratio measured in FB in case 7 (0.97) was higher than in other cases (maximum: 0.1) it was substantially lower than in the muscle. Importantly, the stability of cocaine in muscle should be increased after death as a result of the rapid post-mortem decline in pH that occurs in this tissue (declining to around pH 5.7 – 6.0).

Investigation of the *in vitro* stability of cocaine revealed a remarkable increase in the drugs stability in muscle tissue as compared with horse blood (Chapter 4). Cocaine and BZE concentrations measured in human muscle should, therefore, reflect more closely those at the time of death than they do in blood. Thus, muscle not only has potential value in the interpretation of cocaine and metabolite concentrations measured in fresh cadavers, but it may also provide a substantially more reliable tissue than blood in cases with an extended interval between death and sampling.

The findings of this study indicated that cardiac muscle (CM) might also provide a specimen in which cocaine and metabolite concentrations may be interpreted. Observations made in CM corresponded to those in ThM with ThM/CM cocaine ratios typically around 0.8. The concentration of all analytes in CM correlated well with ThM and FB. Owing to the relatively high uptake of cocaine by the heart, despite the rapid rate at which cocaine is

cleared, it might be expected that cocaine concentrations in this muscle would be significant, especially in chronic users or in individuals who have consumed large amounts of the drug (Karch 2009). In one case analysed in the present study (case 3) the concentration of cocaine in CM (0.73 mg/L) was markedly higher than in ThM (0.35 mg/L). Such an observation would suggest that cocaine accumulates in cardiac muscle and it may be that a very high concentration of cocaine in CM relative to that in ThM could provide an indicator of chronic use or a recent consumption of a large cocaine dose. Given that cocaine is cardio-toxic (Karch et al. 1995), data on the accumulation of cocaine in CM is of clinical and forensic importance yet concentrations in this tissue are rarely reported in the literature. In two cases of acute cocaine overdose Poklis et al. (1987) reported the cocaine concentration in cardiac muscle (myocardium). In one of these cases the authors reported comparable concentrations in myocardium (6.1 mg/kg) and skeletal muscle (6.1 mg/kg), which is in accordance with observations made in the majority of the present cases. In the other case only the myocardium concentration was measured (5.3 mg/kg). In total only five cases were analysed in the Poklis study and in only three of these were muscle concentrations reported. These findings are further limited because the site of blood collection was not stated, although since the work was published prior to the general acknowledgment of post-mortem redistribution it is highly likely that blood was collected from the heart. As with the Garriott study there was no mention of the analytical method having been validated in any matrix prior to use.

Owing to the apparent lag in the distribution of cocaine into the VH, this fluid has been reported to be of use in the interpretation of acute cocaine deaths (Antonides et al. 2007). These authors reported that in cases in which acute cocaine intoxication was apparent just prior to death the concentration of cocaine in the blood exceeded that in the VH and the BZE concentration in the blood was as much as 10-fold higher than in the VH. The opposite situation was observed in the present study; the VH/FB ratios were very high (mean: 13; range: 2.8 – 27; SD: 9.4) even in the case where a short survival time was suspected (case 7; VH/FB ratio: 2.8), and the concentration of BZE was higher than or comparable to that measured in the blood. These observations were in accordance with those made in muscle and considering the evidence presented by Antonides et al. (2007), further support the theory of prolonged survival time and/or, particularly in case 7, repeated

dosing prior to death. The mean concentration of cocaine in the VH was significantly higher than in the blood and in the ThM. Such high VH cocaine concentrations relative to those in muscle would not necessarily have been expected given the findings of the *in vitro* stability study (Chapter 4). The overall order of cocaine stability in unpreserved animal samples was muscle >> VH > blood; the stability of cocaine in sheep VH was substantially less than in deer muscle. Since both tissues lack esterase activity this observation could be attributed to the higher pH of the vitreous. It was proposed that whilst the higher concentrations of cocaine in VH compared with muscle from real cases could, in part, be explained by differential blood flow to these tissues, post-mortem diffusion of cocaine into the VH might also be a contributory factor. Studies have reported that the concentration of cocaine increases in the VH during the post-mortem interval (Beno and Kriewall 1989; Hearn et al. 1991b; McKinney et al. 1995). In a human decedent the concentration was observed to increase by 350% within 18 hours (Hearn et al. 1991b). It was speculated in the present study that post-mortem increases in cocaine concentration would also explain why the cocaine to BZE ratios were typically higher in the VH; when excluding case 7 the mean cocaine/BZE ratio in VH was 0.52, and was found to be significantly higher than in the muscle (mean: 0.14). The opposite might be expected given that the chemical hydrolysis of cocaine at the higher pH of the VH should be more rapid than in the muscle. Despite the potential for rapid post-mortem changes, cocaine and metabolite concentrations in this study were well correlated between FB and VH and in case 7 the cocaine to BZE ratio (2.2) was comparable to that measured in thigh muscle (2.7) and thus indicative of short survival time in this case. These findings, combined with the increased likelihood of detecting cocaine in this matrix (in one case cocaine could only be detected in the VH), indicate that the VH could provide a useful specimen for toxicological interpretation in cocaine related deaths, particularly given its previously reported utility in determining acute cocaine deaths (Antonides et al. 2007).

It is generally accepted that isolated blood measurements have little interpretive value in cocaine determinations. The findings of the present study are in agreement with this and indicate that toxicological assessments made in muscle tissue, and perhaps VH, can increase the likelihood of a reliable interpretation, especially when considered in relation to measurements made in blood. Given that the concentration of cocaine and its metabolites

measured in the *rectus femoris* are apparently independent of the within-muscle sampling location and considering the excellent correlation observed between muscle and blood at the time of death, it can be concluded that muscle may be an important specimen for interpretation in cocaine related death. Further, as a consequence of the increased post-mortem stability of cocaine in muscle, this tissue could have greater interpretive value compared with blood, particularly in cases with an extended period between death and tissue sampling.

8.2 The tissue distribution of opiates

A major contribution of this work is that it has supplemented the existing opiate distribution data for FB and VH in a large sample number. Unlike most studies which have examined the relationship between FB and VH without consideration of the case circumstances, this study has assessed relationships between these matrices according to rapid and delayed death in addition to the relationship observed when including all cases.

Graphical presentation of the distribution of morphine (Figure 26), codeine (Figure 27) and 6AM (Figure 28) clearly illustrated the different distributions of the three analytes between the FB and VH observed in this study.

In agreement with previously published data (Pragst et al. 1994; Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007), the concentrations of morphine in the VH in this study were typically lower than in blood. This observation has been attributed to incomplete distribution in cases with short survival time and the relatively high plasma protein binding of morphine leading to smaller equilibrium concentrations in VH (Pragst et al. 1994; Pragst et al. 1999). The present findings support both theories because 1) the relative concentrations of morphine in VH and FB were influenced by survival time, with much higher VH/FB concentration ratios observed in delayed deaths and 2) codeine, which is not as highly protein bound as morphine, had consistently higher concentrations in the VH compared with the blood.

The findings of the present study were in agreement with those of Pragst et al. (1994) in that there was a trend toward lower VH/FB ratios in cases in which the survival time was short. In 85% of deaths that were either known to be rapid, based on witness and/or scene

information, or could be interpreted as rapid based on the presence of 6AM in blood ($n = 33$), the VH/FB morphine ratio was < 1.0 . In the remaining 15% of cases with a short survival time this ratio exceeded 1.0 with a maximum of 1.7. Based on these findings it was postulated that a high VH morphine concentration relative to that in the blood might also reflect accumulation of morphine in the VH. Owing to the absence of glucuronidating enzymes in the VH the half-life of morphine in this matrix would be expected to be longer than in blood and thus with repeated use prior to death might result in accumulation in the VH relative to the blood. Although VH/FB ratios were particularly high in some delayed deaths (maximum: 5.2) in a few delayed deaths the VH/FB ratio was very low (< 0.4) and a short survival time might have been inferred based on this ratio. In light of this, the assessment of survival time based on the relative concentration of morphine in FB and VH would not be recommended.

In this study morphine was found to be highly correlated between the FB and VH for all cases ($r_s = .782$; $p < .01$; $n = 70$). Stephen et al. (2006) also reported a high correlation between FB and VH free morphine ($r = .716$; $p < .001$) in 52 heroin related deaths and concluded that in cases where a blood sample was unavailable for analysis the VH morphine concentration could be used to predict the concentration in a blood sample. The findings of the present study demonstrate that despite a high correlation such inferences cannot be made from VH. The fact that the relative concentration of morphine in FB and VH in the present study were highly dependent on survival time and given that they may also be further influenced by accumulation following repeated use, it may be concluded that in the absence of a blood sample the blood concentration cannot be inferred from that measured in the VH. The potential for wide variation in the relative concentrations of morphine in FB and VH has been evidenced by Gerostamoulos and Drummer (1997) who, using a validated analytical procedure, reported that there was no correlation between VH and FB morphine ($r = .261$) in 40 cases of heroin related death.

High 6AM concentrations in VH relative to blood have been consistently reported and have been attributed to the lack of esterase activity in VH resulting in increased 6AM stability in this matrix (Pragst et al. 1999; Wyman and Bultman 2004; Antonides et al. 2007). The importance of the VH for discerning heroin exposures through 6AM detection was

highlighted in the present study. In 56% of cases the use of heroin prior to death would not have been confirmed without the assay of the VH. Discerning heroin use is of great importance in forensic toxicological interpretation of opiate related death and as such the extended window of detection the VH provides for 6AM is a principal value of this tissue over blood.

The more frequent detection of 6AM in VH and the substantially higher concentrations in this matrix compared with blood is indicative of increased 6AM stability in the VH. However, the findings of the *in vitro* stability study contradicted this. In unpreserved sheep VH stored at 4°C a loss of 52% 6AM was observed by day 7, which was comparable to the loss observed in horse blood. A previous *in vitro* study demonstrated that 6AM was stable in post-mortem human VH for 7 days at 4 and 0°C (Pragst et al. 1999). It was speculated in the present study that the apparently poor stability of 6AM may be due to bacterial contamination of the animal VH. This theory was supported by the significant increase in stability of 6AM that was observed in VH preserved with NaF at all storage temperatures. These findings do not support previous reports that neither animal nor human VH supports bacterial growth (Davey et al. 1987; Egger et al. 1997). In support of the present findings, a previous study reported that after one year of storage at -18°C, 6AM was only detectable in human VH specimens that had been stored with fluoride (Holmgren et al. 2004). It may be postulated that the VH could lose sterility once taken out of the body and unless stored correctly 6AM can break down substantially in this matrix during a short storage period. It may be concluded that the addition of fluoride to VH is essential as this will increase the likelihood of detecting heroin exposures, particularly since toxicological analyses may not be carried out for several days or much longer following receipt of specimens.

The detection of 6AM in blood is regarded as a reliable indicator of short survival time following heroin intake (Cone et al. 1991; Goldberger et al. 1994; Burt et al. 2001). The present findings are in agreement with this. The absence of 6AM in blood cannot, however, be regarded as an indicator of an extended survival period. In 13 rapid deaths 6AM could only be detected in the VH in the present study. A further finding of the present study was that the detection of noscapine in blood was associated with rapid death. Such an association might be expected owing to the extensive metabolism of noscapine in blood

(Moffat et al. 2004), yet the utility of this compound in assessing survival time has barely been reported. Taylor and Elliot (2009) reported that in cases in which rapid death could be implied from the scene, i.e. needle still in arm or tourniquet still around arm, opium poppy constituents could be detected in the blood. The blood detection of noscapine may be significant in the interpretation of acute heroin deaths because in the present study it was detected in two rapid death cases in which 6AM could only be detected in the VH. The current utility of noscapine is reported to be the urinary detection of its primary metabolites for identifying street heroin use in patients prescribed diamorphine (McLachlan-Troup et al. 2001; Paterson et al. 2005; Trathen et al. 2009). This study has demonstrated that the blood detection of noscapine provides a valuable indicator of short survival time in heroin-related deaths, particularly since it may be detected over a longer timescale than 6AM.

In agreement with several previous reports, the present study demonstrated an association between survival time and the FM/TM ratio in blood. The mean FM/TM ratio in rapid deaths (0.55) and delayed deaths (0.26) were comparable to previous results reported by Spiehler and Brown (1987), Staub et al. (1990) and Goldberger et al. (1994). However, these previous studies either utilised cardiac blood or they did not specify the site of blood collection. Although many studies have advocated the use of the FM/TM concentration ratio in assessing survival time, some authors advise against this owing to the many factors that can affect the FM/TM ratio, particularly during the post-mortem interval (Skopp et al. 1996; Karch 2009). In the present study the assessment of survival time based on the FM/TM ratio would have led to erroneous conclusions in two cases where very low ratios were observed in cases where 6AM could be detected in the blood. Low FM/TM ratios in cases with a short survival time may reflect accumulation of morphine glucuronides in regular heroin/morphine users (Spiehler and Brown 1987; Tollison et al. 2002) or in individuals with renal insufficiency (Faura et al. 1998). Thus, a low FM/TM ratio may be observed despite very recent heroin exposure. In agreement with the findings of Spiehler and Brown (1987), a high FM/TM was not maintained in any of the delayed death cases analysed in the present study (maximum FM/TM ratio: 0.39). However, a high FM/TM might be expected if a person was deficient in glucuronidating ability regardless of survival time (Spiehler and Brown 1987). Given the many factors that can affect the FM/TM ratio

(Section 2.4.2 and 7.6), caution should be employed when interpreting this ratio, particularly when considered in isolation of other toxicological information.

The concentrations of morphine observed in the present study were typically low with 79% of cases having a blood concentration less than 0.3 mg/L. In only some of the present cases could these low concentrations be attributed to extended survival periods, with many rapid death cases also having morphine concentration < 0.3 mg/L. These findings are in agreement with numerous reports in which the concentrations of morphine associated with heroin related deaths were observed to be lower than or comparable to those observed in living users and in users dying of causes other than overdose (Monforte 1977; Kintz and Mangin 1995; Zador et al. 1996; Fugelstad et al. 2002; Darke et al. 2007). When not associated with extended survival periods, low morphine concentrations have been attributed to the concomitant use of other drugs and/or loss of tolerance (Ruttenber and Luke 1984; Spiehl 1989; Fugelstad et al. 2002; Verger et al. 2003; Darke et al. 2007; Farrell and Marsden 2008). The prevalence of polydrug use was very high in the present study, particularly involving alcohol and diazepam. In only one case was morphine detected in the absence of another drug substance (excluding codeine and 6AM). In contrast to previous reports (Ruttenber and Luke 1984; Steentoft et al. 1988; Ruttenber et al. 1990; Fugelstad et al. 2002; Darke et al. 2007) there was no significant negative association between blood morphine and alcohol concentration in the present study. The findings of this study were consistent with reports that alcohol use increases the risk of heroin overdose (Ruttenber and Luke 1984) because alcohol detection and alcohol concentrations > 100 mg/dL were associated with rapid death. Given the potent respiratory depressant effect of opiates and the difficulty associated with determining an individual's opioid drug tolerance, determining the relative contribution that a concomitant drug makes to fatal opioid overdose remains extremely difficult.

The utility of several toxicological indicators of survival time have been discussed based on the findings of this study. Frequent overlap in the expected values in rapid and delayed deaths means that indicators may not be reliable on a case by case basis. Despite the excellent correlation between VH and FB morphine, it can be concluded that in the absence of a blood sample, the blood concentration cannot be inferred from that measured in the

VH. Nevertheless, assessments based on the combined toxicological information obtained in FB and VH should greatly assist in the interpretation of heroin/morphine fatalities. The VH provides a useful adjunct to interpretation owing to the extended window of detection it provides for 6AM, providing that samples that cannot be analysed promptly are preserved with NaF. Whilst a very high morphine concentration may be consistent with the consequences of heroin toxicity, notwithstanding the issue of tolerance, the interpretation of low concentrations requires the consideration of more factors. Even with further research into this field, the many factors known to affect the concentrations of drugs and their distribution, particularly during the post-mortem interval, the interpretation of drug concentrations in opiate related-deaths is, and will most likely remain, extremely challenging. In answer to the vital question, 'did heroin/morphine cause or contribute to death?', the answer is likely to come from a combined analysis of the toxicology, medical/drug history, the reported circumstances of death, the role of other drugs and possible pharmacokinetic factors, an assessment of tolerance, and the exclusion of other causes.

8.3 Limitations and future work

One of the main limitations of the present work was that the opportunity to examine the distribution of opiates in muscle did not arise and this meant that one of the main objectives could not be addressed. The muscle distribution study was carried out at the Medico-legal Institute, Sao Paulo (Brazil), where heroin/morphine involved deaths are relatively rare and none were known or suspected during the time of sample collection. Previous work has suggested that muscle may be a suitable specimen for morphine analysis with concentrations measured in muscle being comparable to blood (Garriott 1991). It has also been suggested that muscle may provide a good specimen for roughly predicting the FM/TM ratio in blood (Moriya and Hashimoto 1997). In a single case of rapid death following injection of heroin and methamphetamine, these authors reported FM/TM ratios of 0.86, 0.74 and 0.79 in the FB, femoral muscle and cardiac muscle respectively. Whether morphine is subject to intra- and inter-muscular variation has not been reported previously and thus a systematic investigation of this is required to elucidate the interpretive value of muscle in opiate determinations. The importance of such knowledge is highlighted by the

case of Dr Harold Shipman (Pounder 2003), where toxicological analyses had to be conducted following exhumation and where only muscle tissue and liver were suitable for analysis. As the liver is the site of first pass metabolism, morphine concentrations measured in this tissue are of little interpretive value other than to indicate that the drug has been taken. Free morphine and TM could be detected in the muscle of 11 exhumed bodies, some of which had been embalmed. Owing to the limited understanding of how morphine concentrations in muscle relate to those in blood and how these concentrations change in muscle over time, i.e. to what extent morphine glucuronides deconjugate in the muscle during the post-mortem interval, the prosecution's case against Dr Shipman relied more heavily upon the circumstantial evidence (Pounder 2003). Further work on the distribution and post-mortem stability of opiates in muscle would greatly assist in future cases where an interpretation of opiate concentration in muscle is required.

Data on the concentration of 6AM in muscle tissue does not appear to have been reported in the literature but owing to its high lipophilicity this metabolite should distribute into the muscle. The increased *in vitro* stability of 6AM in deer muscle compared with horse blood observed in the present study suggests that this tissue might, like VH, provide a prolonged window of detection for this forensically important metabolite. This could be of particular use in cases where blood and VH are not available, i.e. in decomposing bodies. Given the low detection limits that are generally possible today, even in solid tissues, it seems unlikely that the lack of 6AM data in muscle is due to low concentration in this tissue. It is more likely that 6AM is infrequently assayed in muscle. Comparison of blood, VH and muscle tissue 6AM concentrations in heroin fatalities would provide information on the relative *in vivo* stability of this metabolite and possibly reveal a utility of muscle in the determination of heroin exposures.

The major limitation with the cocaine distribution study was the small number of cases that were available for analysis ($n = 9$). A further limitation was the lack of information that was available pertaining to the circumstances surrounding these cases. In only one case was a short survival time suspected. Consequently, the reliability of thigh muscle in assessing survival time cannot be confirmed from the findings of this study. Nevertheless, the preliminary results suggest that this tissue certainly has potential in making such a

determination. The unavailability of cases in which death was due to acute cocaine overdose meant that the utility of muscle in distinguishing between cocaine induced death and death resulting from other causes could not be assessed. Although a short survival time was suspected in one case (case 7), death as a direct result of cocaine could not be confirmed in this case. It is thought that the cocaine/BZE ratio in brain provides an indicator of lethality and survival time in cocaine-related death; brain is believed to have superior interpretive value compared with other tissues and blood. Whether muscle can be of similar interpretive value to brain requires further investigation in cases where detailed case information is known. More data is also required in cases with varying circumstances of death in order to clarify whether the uniform distribution observed in this study is a feature of repeated cocaine use leading to accumulation and equilibration or whether it is more dependent on the survival period. It is probable that both factors affect distribution. Information pertaining to drug history and/or frequency of drug use prior to death was not available in these cases. Hair was collected at autopsy in most cases with the hope of conducting segmental analyses although the length of the hair was not sufficient to allow more than one analytical determination in any case. Since a single dose of cocaine (25 – 35 g) can be measured in hair for 2 – 6 months (Karch 2009), the chronicity of use cannot be inferred from the concentration measured in a single segment. Additional information from witnesses, family or medical practitioners would have greatly assisted the interpretation of the drug distributions observed in these cases.

Given the reported potential for inter- and intra-muscular variation in drug concentration (Williams and Pounder 1997; Langford et al. 1998), future studies should clearly define the muscle sampling site so that drug concentration data from individual muscles can be compared across the literature. Evaluating trends from the published data to aid in the interpretation of cocaine and its metabolites in this matrix is otherwise impossible. Following the introduction of the Human Tissue Act, examining drug distribution in tissues that are not conventionally collected at autopsy is extremely difficult. However, the collection of non-conventional tissues, such as muscle, may be met with less controversy providing that such tissues can be shown to be of interpretive value in toxicological determinations. This highlights the importance of publishing findings such as those presented in this thesis. In the determination of cocaine and its metabolites the *rectus*

femoris muscle potentially provides a reliable specimen that can be sampled with ease at autopsy owing to its convenient mid-anterior location. Sampling from multiple locations within this muscle is recommended so that drug distribution may be assessed on a case by case basis. The collection of multiple small samples within a single muscle or within a few different muscles can certainly be justified in cases of drug-related death because potential variation in drug concentration can seriously affect case interpretation. Published series of autopsy data where case circumstances are known would certainly aid in elucidating the interpretive value of muscle in drug-related deaths and may see muscle become a routine toxicological specimen for this purpose, particularly in the analysis of cocaine and its metabolites.

Uncertainty in the interpretation of the relative concentrations of morphine in VH and FB largely resulted from the lack of published information pertaining to the accumulation of morphine in this fluid. It was postulated in this study that VH/FB morphine ratios that were unexpected based on the known survival time, i.e. high VH/FB ratio in cases with short survival time, could be due to accumulation in the VH. However, accumulation of morphine in the VH has not been reported and this is an area that requires further investigation. Future work should aim to elucidate the relationship between morphine concentration in the FB and VH following repeated heroin/morphine use prior to death in cases with varying survival times. It would be of use to know if this relationship is different to that observed in naive users or in regular users dying following a single dose of heroin/morphine. It may be that a high VH/FB ratio is characteristic of repeated use prior to death in addition to its association with an extended survival period. If this relationship can be better elucidated the VH/FB morphine ratio might be of interpretive value in opiate-related deaths.

A further factor which might have limited the opiate findings in the present study is that the time interval between death and sample collection was much longer than in the cocaine cases. Although in the majority of cases the bodies were refrigerated within hours following death, which would have slowed the progression of putrefaction, post-mortem changes in drug concentration cannot be ruled out. The time interval between death and post-mortem examination in these cases varied from between 1 day to 6 days. Whilst it well known that

changes in morphine concentration might occur in blood, i.e. post-mortem hydrolysis of the glucuronides and redistribution of morphine from tissues to blood (Moriya and Hashimoto 1997; Skopp 1997), very little is known about changes that might occur in the VH. As a consequence of its isolated location within the eye, it might be expected that the VH would be less affected by post-mortem change compared with the blood, although this certainly is not the case for cocaine (Beno and Kriewall 1989; Hearn et al. 1991b; McKinney et al. 1995). There is currently no data in human subjects relating to post-mortem changes in opiate concentrations in VH. In a porcine model, concentrations of morphine in the VH were reported to increase within the first hour after death and then to slowly decline thereafter (Crandall et al. 2006b). The observed changes were minor compared to that observed for cocaine, implying that morphine concentrations are more stable in the VH than cocaine. The literature would benefit from more studies on the temporal concentration changes occurring in VH in the early post-mortem period, such as those that have been conducted for cocaine. Although complicated by restrictions imposed by the Human Tissue Act, ideal investigations would involve studying human subjects. If further research can confirm that changes in morphine concentration occurring in the VH after death are not severe this would further promote the use of VH for opiate interpretation.

Chapter 9.0 Thesis Conclusions

This research focused on the distribution and stability of opiates and cocaine in various post-mortem tissues. The study critically reviewed available literatures relevant to the aim and orientation of this research (Chapter 2). It also developed and validated sensitive and specific methods appropriate for measuring drug and metabolite concentrations in the chosen tissues (Chapter 3). The *in vitro* stabilities and factors affecting storage of the chosen drugs were examined (Chapters 4, 5). The relationship between the distribution of these drugs in less conventional tissues (muscle and vitreous) and blood was also assessed (Chapter 6 and 7). The significance of toxicological findings, in terms of assisting with toxicological interpretation, was also assessed (Chapters 6, 7 and 8).

This work revealed the following novel aspects of those drugs in relation to post-mortem and forensic investigations.

- 1) cocaine and its metabolites were evenly distributed throughout thigh muscle bulk;
- 2) muscle concentrations were much higher than in blood and correlated with the blood at the time of death;
- 3) the stability of cocaine was increased substantially in deer muscle (as compared with horse blood and sheep VH) indicating that the concentration measured in human muscle may reflect more closely the concentration at the time of death than blood providing samples that cannot be analysed promptly are stored at 4°C or below;
- 4) the cocaine to BZE ratio measured in muscle and VH may be of interpretive value in the assessment of survival time;
- 5) fluoride preservation (2%) and frozen storage was essential for the stability of cocaine and 6AM in horse blood
- 6) fluoride preservation (1.5%) and frozen storage was essential for the stability of 6AM stability in VH
- 7) the concentration of morphine in blood cannot be inferred from that in the VH

Based on the work undertaken the following protocol for specimen collection, storage and results interpretation could be used in cases of opiate- and cocaine-related death. However,

the possibility that variable results will be obtained depending on the circumstances of death cannot be excluded.

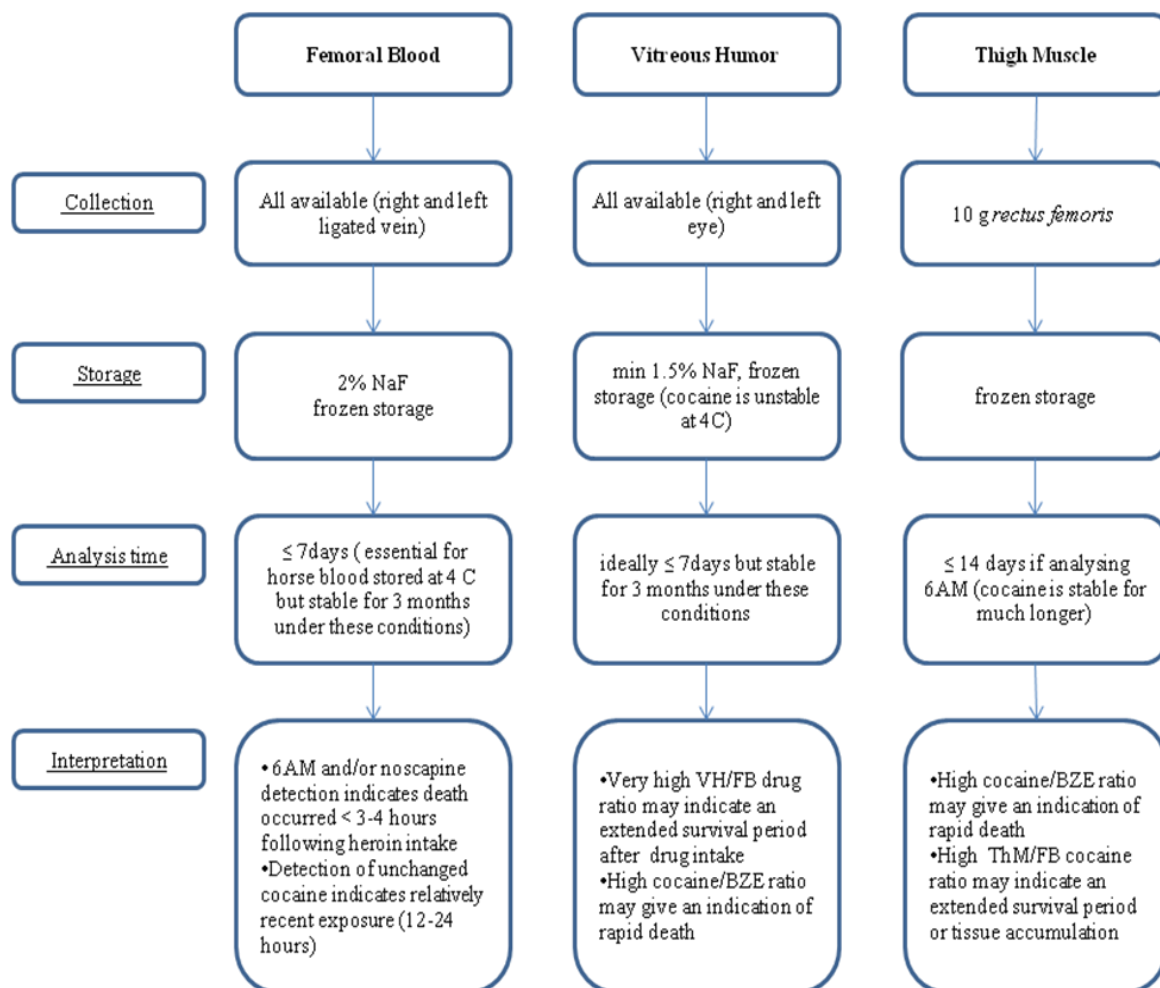


Figure 30 Ideal pathway of autopsy and toxicology protocol

This research has identified some of the limitations and suggests possible areas for future research. Muscle is potentially interpretive but has not been sufficiently characterised in the present study due to limited cases and this could form the basis for future studies. In particular, additional distribution data is required for cocaine and opiates in muscle tissue so that the interpretive value of this tissue may be further elucidated.

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Abbreviations

6AM	6-acetylmorphine
6AM-d3	Deuterated 6-acetylmorphine
AB	Afro Brazilian
AChE	Acetylcholinesterase
AMD	Automatic method development
amitrip	Amitriptyline
β-gluc	β-glucuronidase
BAC	Blood alcohol concentration
BBB	Blood brain barrier
BMI	Body mass index
BPM	Buffer preserved muscle
BSTFA	Bis-(trimethylsilyl)-trifluoroacetamide
BuChE	Butyrylcholinesterase
BZE	Benzoyllecgonine
BZE-d3	Deuterated benzoyllecgonine
C/FM	Codeine to morphine concentration ratio
CB	Cardiac blood
CE	Carboxylesterase
CID	Collision induced dissociation
citalop	Citalopram
CM	Cardiac muscle
CNS	Central nervous system
COC	Cocaine
COET	Cocaethylene
CSF	Cerebrospinal fluid
CYP (3A4, 2C8, 2D6)	Cytochrome P450 (3A4, 2C8, 2D6)
δ	Delta
D1 D2	Dopamine receptors
DHC	Dihydrocodeine
df	Degrees of freedom
dL	Decilitre
Dz	Diazepam
E	Ethanol
<i>E. coli</i>	<i>Escherichia coli</i>
ECG	Ecgonine
ED	Excited delirium
EEE	Ecgonine ethyl ester
EI	Electron ionisation
EME	Ecgonine methyl ester
FB	Femoral blood
fluox	Fluoxetine
FM	Free morphine
FM/TM	Free morphine to total morphine concentration ratio
GC-MS	Gas chromatography- mass spectrometry
GC-MS/MS	Gas chromatography-ion trap-tandem mass spectrometry
GCR	Glucuronidase
GI tract	Gastrointestinal tract
GSW	Fatal gunshot wound
H	Heroin
<i>H. pomatia</i>	<i>Helix pomatia</i>
hCE	Human carboxylesterase
HCl	Hydrochloride
HPLC-DAD	High-performance liquid chromatography-diode array detection
HQC	High quality control

Ht	Height
ILOQ	Instrument limit of quantification
IM	Intramuscular
IN	Intranasal
IS	Internal standard
IV	Intravenous
κ	Kappa
k (day ⁻¹)	Rate constant
KF	Potassium fluoride
LOQ	Limit of quantification
LQC	Low quality control
M	Molar
m/z	Mass to charge
M3G	Morphine 3-glucuronide
M6G	Morphine 6-glucuronide
MDMA	3,4-methylenedioxymethamphetamine (ecstasy)
meth	Methadone
MG	Morphine glucuronides
min	Minute
mirtaz	Mirtazepine
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
MSTFA	N-methy-N-(trimethylsilyl) trifluoroacetamide
NA	Not available
NaF	Sodium fluoride
ND	No data
NH ₄ OH	Ammonium hydroxide
nosc	Noscapine
NS	No sample
OH	Hydroxyl group
para	Paracetamol
PB	Preserved blood
PBS	Phosphate buffer solution
PM	Preserved muscle
PMI	Post-mortem interval
PMR	Post-mortem redistribution
PVH	Preserved vitreous humour
QC	Quality control
R	Pearson's correlation
R^2	Coefficient of determination
r	Pearson's correlation coefficient
r_s	Spearman's rho correlation coefficient
r_τ	Kendall's tau correlation coefficient
ROA	Route of Administration
rpm	Revolutions per minute
RSD	Relative standard deviation
SD	Standard deviation
SE _b	Standard error of the slope (b)
SIM	Single ion monitoring
SPE	Solid phase extraction
SPSS	Statistical Package for the Social Sciences
τ	Kendall's tau
T _{1/2}	Half life
temaz	Temazepam
THC	Tetrahydrocannabinol
ThM	Thigh muscle

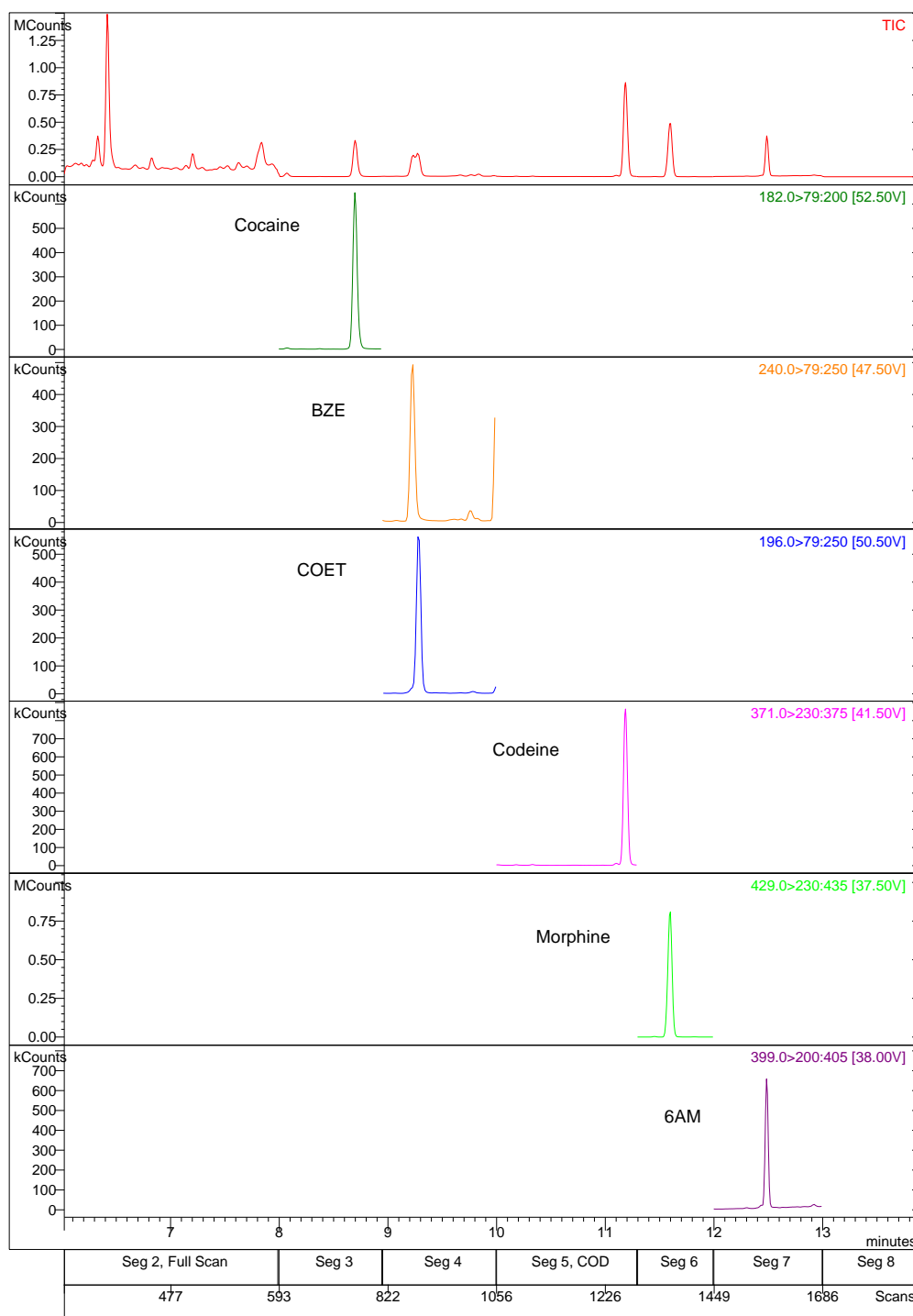
TLC	Thin layer chromatography
TM	Total morphine
TMCS	Trimethylsilyl chloride
TMS	Trimethylsilyl
μ	Mu
U	Enzyme units
UB	Unpreserved blood
UDPGT (2B4, 2B7)	Uridine diphosphate-Glucuronosyltransferase-(2B4, 2B7)
UK	United Kingdom
UM	Unpreserved muscle
USA	United States of America
UVH	Unpreserved VH
V_d	Volume of distribution
VH	Vitreous humour
W	White
w/v	Mass/volume
Wt	Weight
zop	Zopiclone

Appendix 1 Description and optimisation of isolation, dissociation and software parameters

Software Parameter	Description	Optimisation
Scan Time	The duration of each scan (in seconds). The scan time ranges from 0.1 to 5 seconds per scan. The minimum scan time is determined by the selected mass range.	The scan time required adjustment depending on the number of compounds scanned in a single channel. The time had to be reduced when more than one compound was scanned so that sufficient data could be collected for each compound.
Multiplier Offset	The change in voltage applied to the electron multiplier 10^5 gain in order to adjust the sensitivity. The multiplier offset may be increased or decreased by 300 V.	The sensitivity of the MS/MS method was optimised by increasing the multiplier offset to 300 V of the electron multiplier 10^5 gain. In full scan the offset was set at 100 V.
Emission Current	The emission current is a measure of the intensity of the electron beam and in general, increasing the emission current increases the quantity of ions formed in the trap (TIC) for both analyte and background ions. The emission current can be set from 5 to 100 μA .	In MS/MS a high emission current can be used to increase the sensitivity because the MS/MS isolation step eliminates the higher background that would normally arise from a high emission current. For optimum sensitivity the emission current was set to 10 and 50 μA for full scan and MS/MS respectively.
Target Ion Current (TIC) value	The target TIC value determines how many ions enter the ion trap during the ionisation time. If the target TIC is increased, the ionisation time for any given pressure will increase causing the ion trap to create more ions. Increasing the target TIC increases peaks heights but increasing it too far results in a loss of mass resolution. In general, the higher the molecular weight of the compound, the lower the target TIC should be to avoid loss of mass resolution and space charging effects.	A target TIC value for full scan EI operation was set to 20,000. In MS/MS the m/z distribution of the stored ions becomes smaller and fewer ions can be trapped without a loss of mass resolution. An acceptable balance between mass resolution and peak height was observed at a TIC of 10,000 counts for the lower molecular weight cocaine compounds and at 5,000 counts for the higher molecular mass opiates.
Maximum Ionisation Time	This sets the limit for the longest ionisation time AGC can use. The highest maximum ionisation time that can be set is 65,000 μsec .	For both full scan and MS/MS a maximum ionisation time of 25,000 μsec was set for optimum sensitivity.
Isolation Parameter		
Parent Ion Mass	The ion required for isolation (precursor ion). Typically the parent ion but is dependent on stability of the ion. Parent ion mass range is 50 to 650 m/z.	The parent ion stability of COC, BZE and COET under EI conditions was not sufficient for use as precursor ions. The next most abundant ions were chosen for precursor ion isolation.

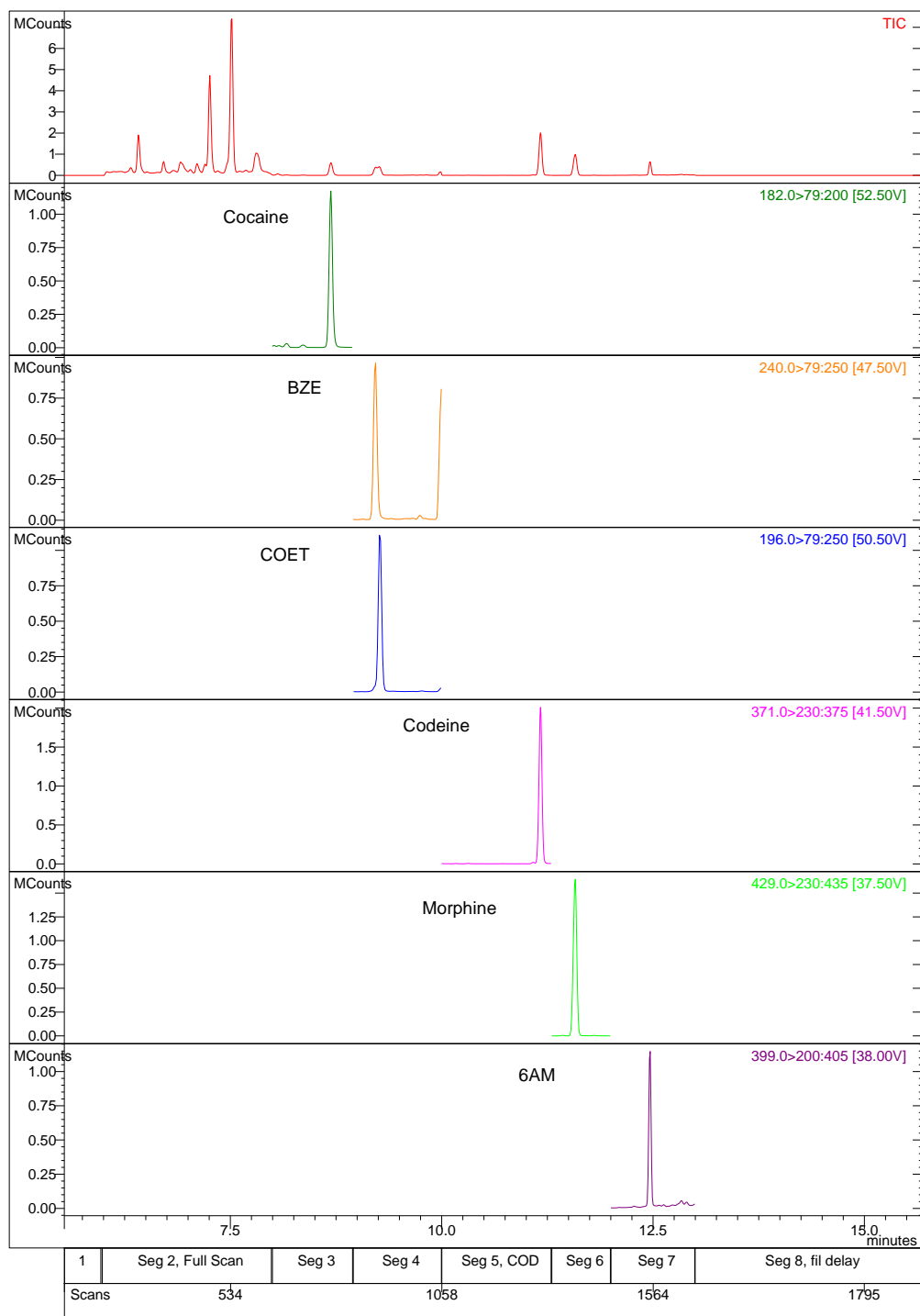
Isolation Window	The isolation window determines the mass range within which ions will be isolated prior to excitation. The full mass isolation window range is 1.0 to 14.0 m/z. Given a parent ion mass of 429 and an isolation window of 3 m/z, ions of masses 428, 429 and 430 would be isolated.	During the parent ion isolation step the isolation window was set to 3m/z to ensure the ion was centred in the window. When analysing standard solutions a window of 3 m/z showed no interference. Following extractions from biological matrices the window was reduced at 0.5 m/z intervals for certain compounds to exclude the isolation of interfering ions.
Dissociation Parameter		
Waveform type	In order to dissociate the parent ion and form a product ion spectrum the kinetic energy of the parent ion is increased by application of a waveform to the trap. Two types of waveform may be applied: <i>Non-resonant excitation:</i> Application of a low frequency dipole field which excites both parent and product ions. <i>Resonant excitation:</i> Application of a high frequency dipole field which matches the oscillation frequency of the trapped ion - it can be selectively tuned to excite only ions having a particular m/z.	MS/MS methods were optimised using both non-resonant and resonant excitation. Whilst resonant excitation gives the purest spectra, only forming first order product ions, non-resonant is the preferred method because, unlike resonant excitation, it is not affected by changes in the trapping conditions or by sample concentration. Since the resulting product ion spectra were almost identical using both methods, indicating that for the compounds investigated non-resonant excitation also only formed first order product ions, the final optimised method used non-resonant excitation.
Excitation Storage Level	The excitation storage level is the rf storage level in m/z when the dissociation waveform is applied following isolation. The excitation storage level range depends on the parent mass, but the storage level must be more than 2 mass units below the lowest product ion value.	Although the Saturn software provides a tool to calculate the storage level based on the parent ion mass, calculated levels did not provide acceptable product ion spectra. A value of 75 m/z, 7 m/z less than the lowest product ion of interest for cocaine and its metabolites, was found to give the best results not only for these compounds but also for the higher mass opiates.
Excitation Amplitude	The amplitude of the applied waveform is called the CID excitation amplitude. The amplitude range for non-resonant excitation is 0 – 100 V and for resonant the range is 0 – 60 V. If the excitation amplitude used is too large, the parent ion and product ion spectra will be absent because both ions will have been ejected from the trap. If the value is too small, the parent ion spectrum will be dominant and the product ion spectrum weak or missing.	The excitation amplitude was increased or decreased until a change in the MS/MS spectra was observed. For non-resonant and resonant 10 V and 0.2 V steps were used respectively. The amplitude was increased or decreased further, but in smaller increments, until suitable ion spectra were obtained. Automatic method development (AMD), with which several voltages can be tested at once, was used to speed up the optimisation process.
Excitation Time	The excitation time is the length of time that the waveform is applied, or, the time required for collision induced dissociation (CID) by ion excitation. The excitation time range is 0 – 1000 msec.	For parent ion isolation, where ion excitation is not required, the excitation time is set to 0 msec. To optimise product ion production the excitation time was adjusted in 10 msec steps. The final excitation time was set to 30 msec for all compounds.

Appendix 2 Total ion current and ion chromatograms of opiates and cocaine in water (VH validation standard)



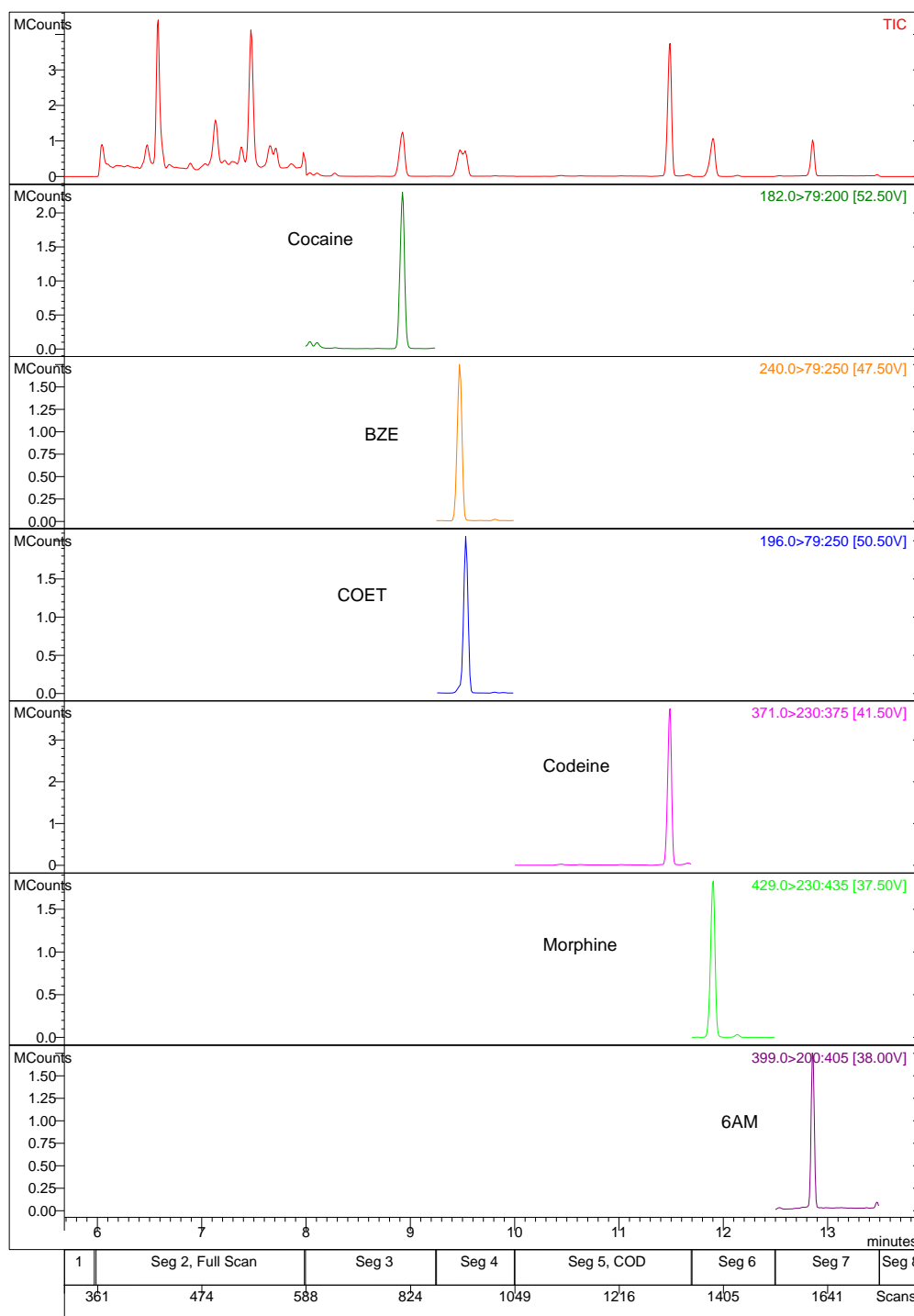
TIC = Total ion current; the extracted quantification ions of cocaine, BZE, COET, codeine, morphine and 6AM are shown in the panes below the TIC.

Appendix 3 Total ion current and ion chromatograms of opiates and cocaine in horse blood
(blood validation standard)



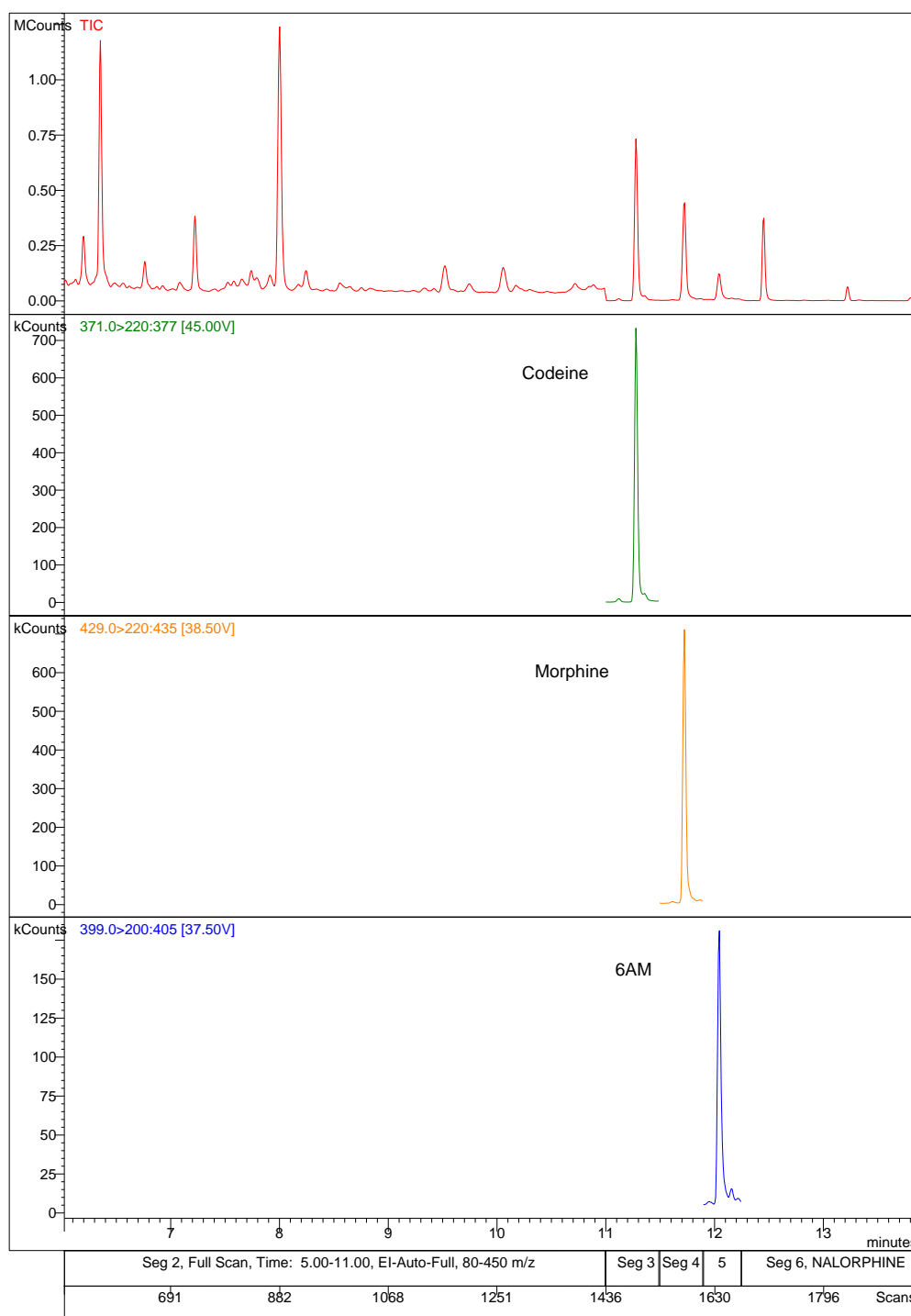
TIC = Total ion current; the extracted quantification ions of cocaine, BZE, COET, codeine, morphine and 6AM are shown in the panes below the TIC.

Appendix 4 Total ion current and ion chromatograms of opiates and cocaine in deer tissue (muscle validation standard)



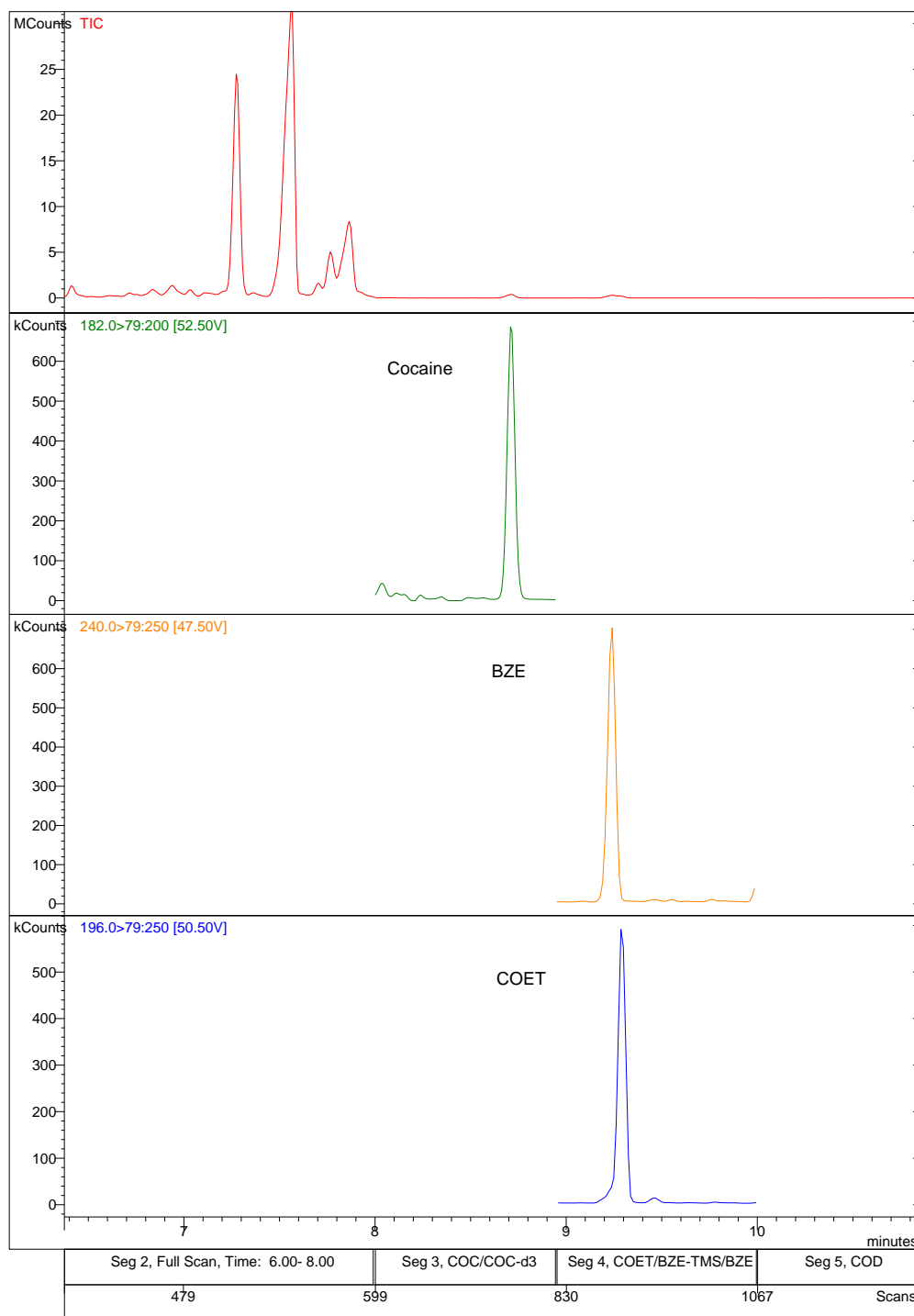
TIC = Total ion current; the extracted quantification ions of cocaine, BZE, COET, codeine, morphine and 6AM are shown in the panes below the TIC.

Appendix 5 Total ion current and ion chromatograms of codeine, morphine and 6AM in vitreous humour from a heroin using decedent



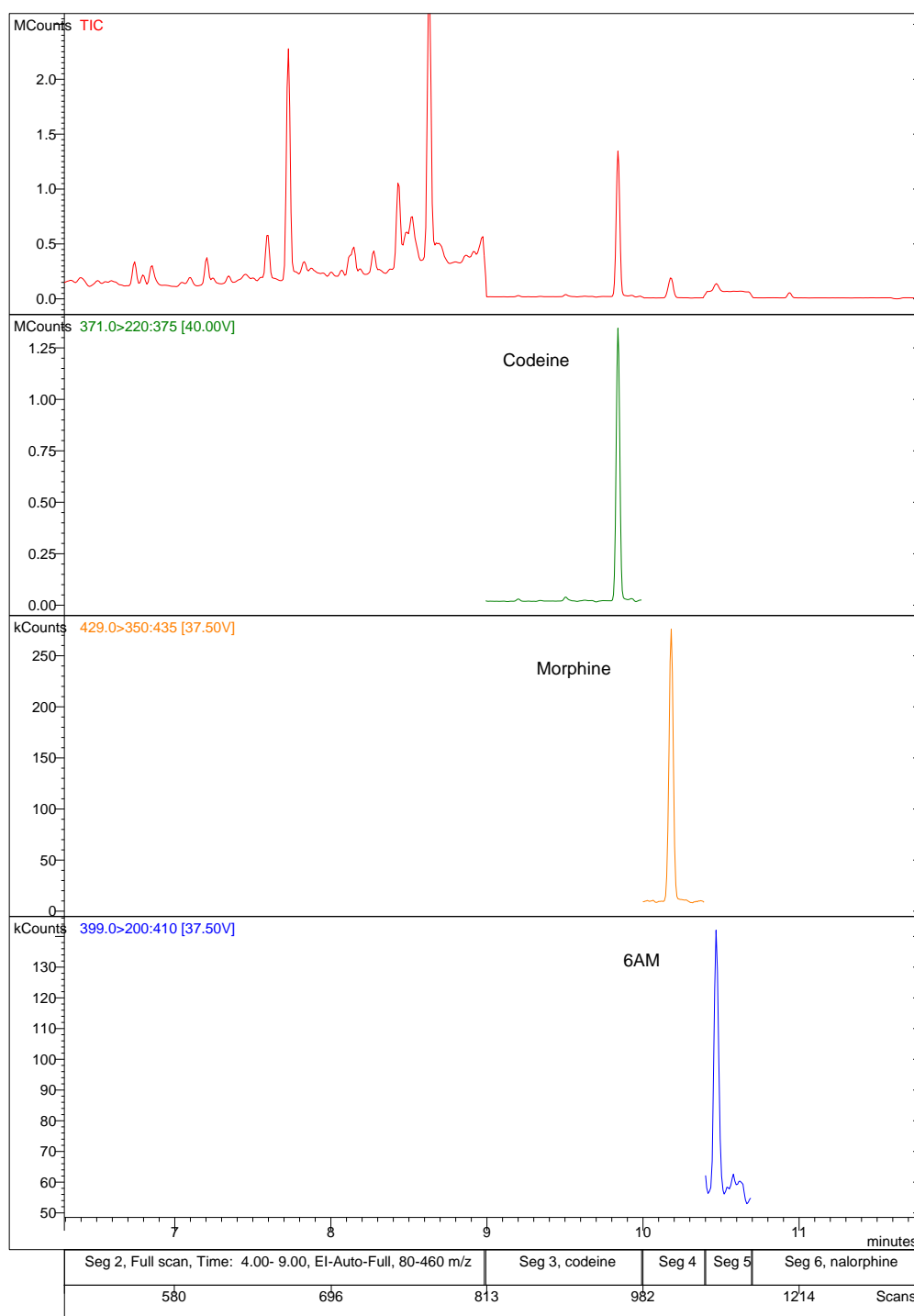
TIC = Total ion current; the extracted quantification ions of codeine, morphine and 6AM are shown in the panes below the TIC.

Appendix 6 Total ion current and ion chromatograms of cocaine, BZE and COET in vitreous humour from a cocaine using decedent



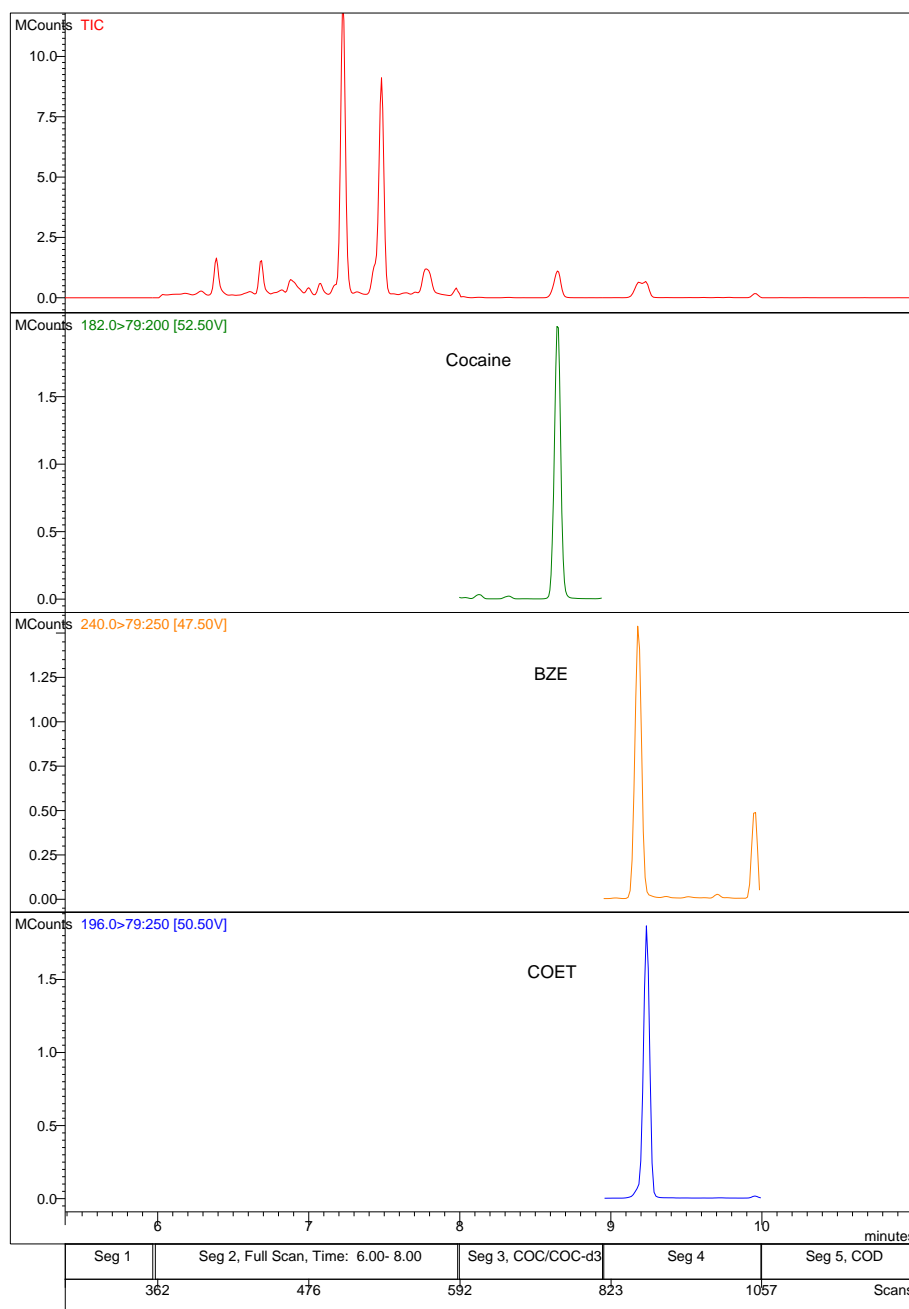
TIC = Total ion current; the extracted quantification ions of cocaine, BZE and COET are shown in the panes below the TIC.

Appendix 7 Total ion current and ion chromatograms of codeine, morphine and 6AM in femoral blood from a heroin using decedent



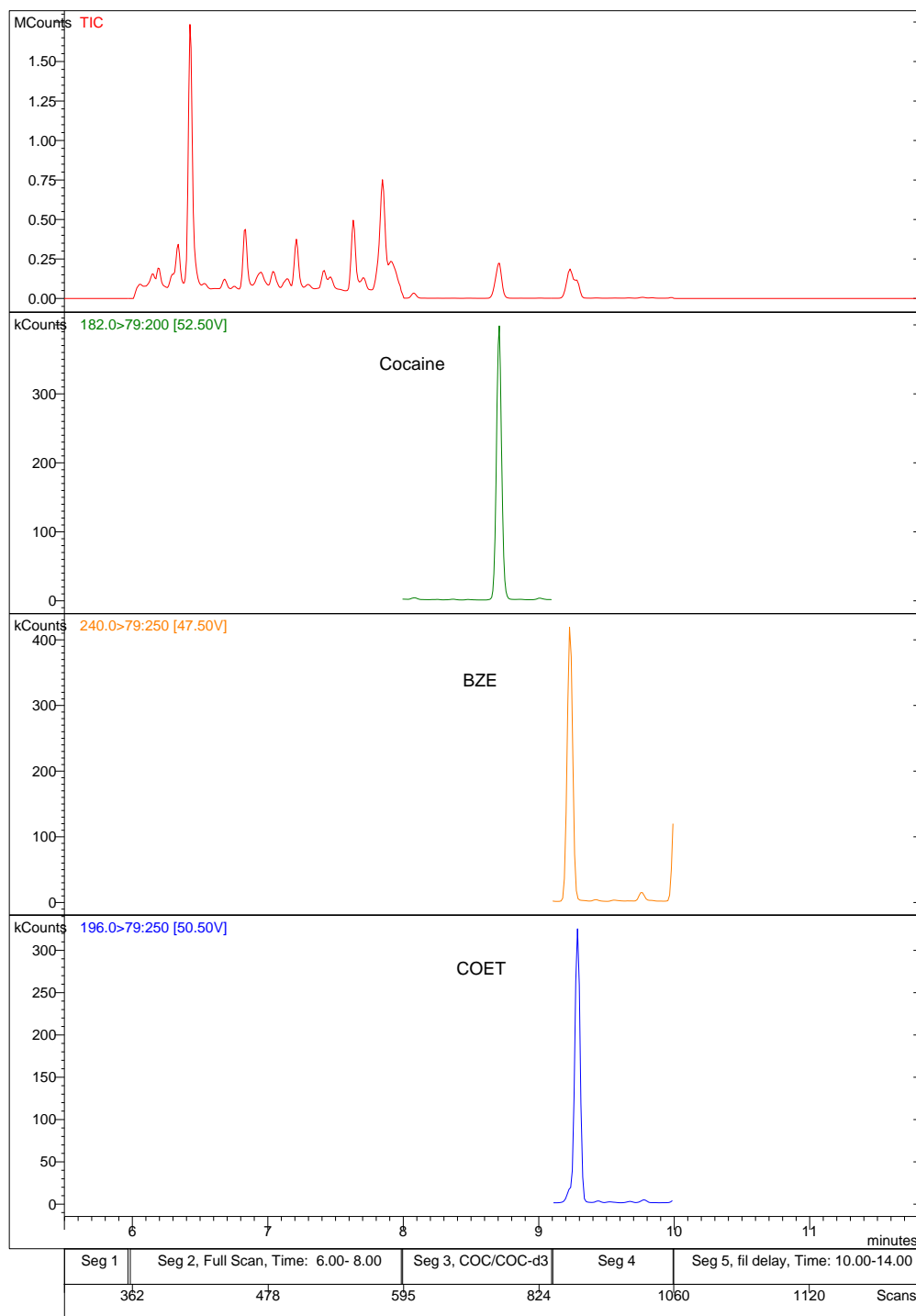
TIC = Total ion current; the extracted quantification ions of codeine, morphine and 6AM are shown in the panes below the TIC.

Appendix 8 Total ion current and ion chromatograms of cocaine, BZE and COET in femoral blood from a cocaine using decedent



TIC = Total ion current; the extracted quantification ions of cocaine, BZE and COET are shown in the panes below the TIC.

Appendix 9 Total ion current and ion chromatograms of cocaine and metaoblites in muscle from a cocaine using decedent

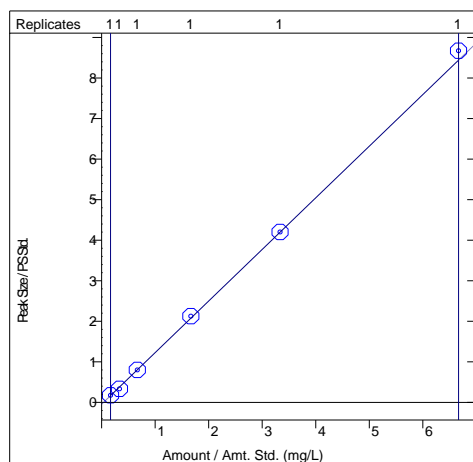


TIC = Total ion current; the extracted quantification ions of cocaine, BZE and COET are shown in the panes below the TIC.

Appendix 10 Aqueous validation: Calibration curves

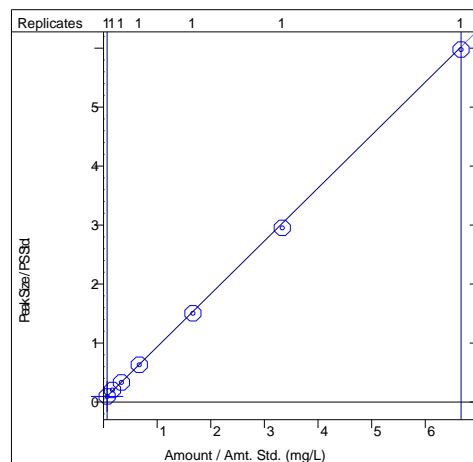
Cocaine

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 10.36%, Coeff. Det.(r2): 0.999822
 $y = +1.2728x - 0.0457$



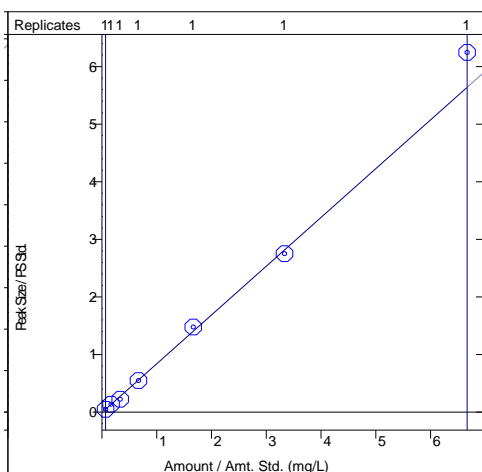
BZE

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 20.11%, Coeff. Det.(r2): 0.999898
 $y = +0.8975x + 0.0381$



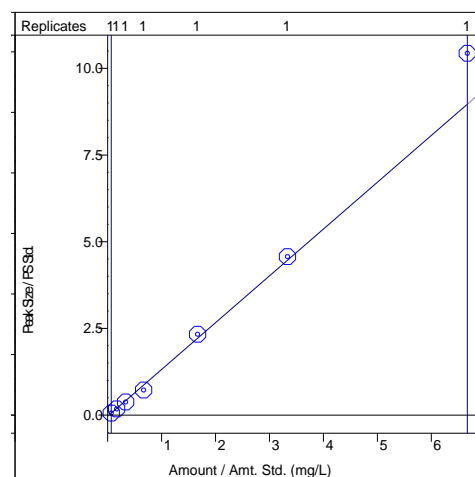
Cocaethylene

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 10.74%, Coeff. Det.(r2): 0.996837
 $y = +0.8472x - 0.0083$



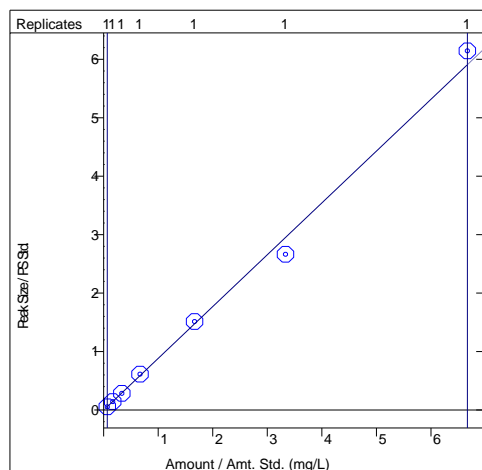
Codeine

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 19.03%, Coeff. Det.(r2): 0.996526
 $y = +1.3499x - 0.0343$



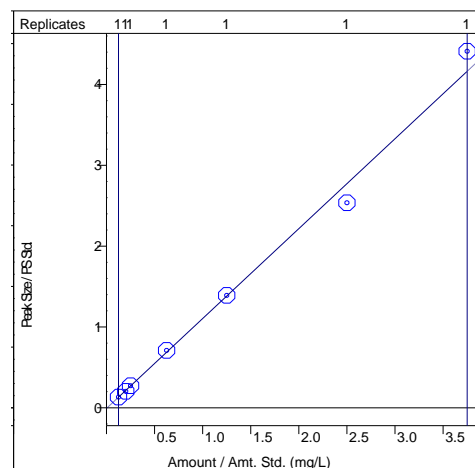
Morphine

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 5.550%, Coeff. Det.(r2): 0.995590
 $y = +0.8867x - 0.0040$



6MAM

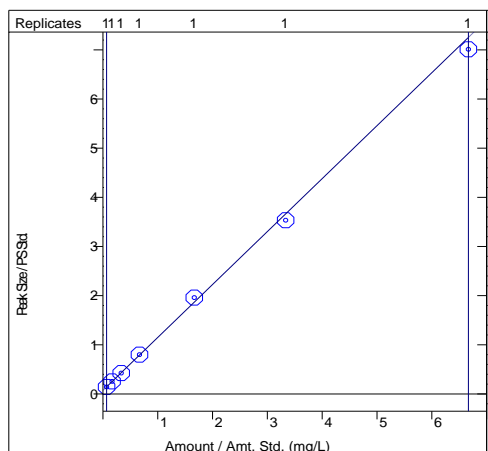
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 5.422%, Coeff. Det.(r2): 0.992786
 $y = +1.1123x - 0.0079$



Appendix 11 Blood validation: Calibration curves

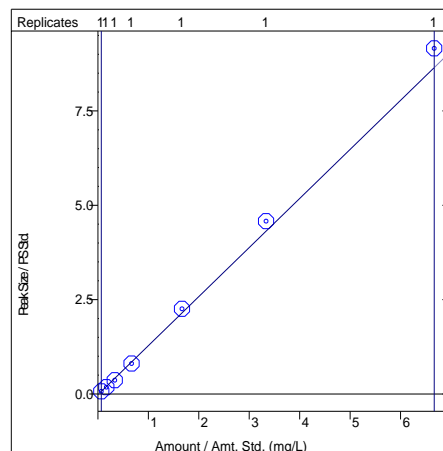
Cocaine

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 28.85%, Coeff. Det.(r2): 0.999502
 $y = +1.0770x + 0.0726$



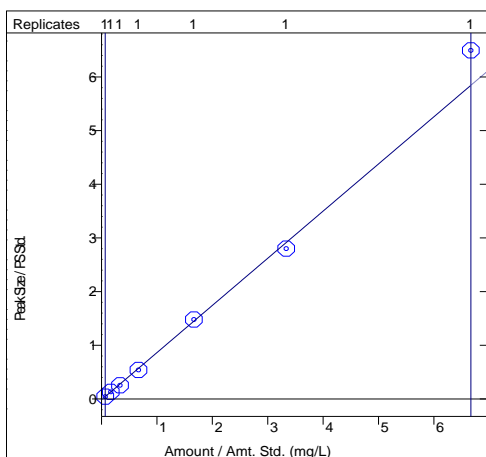
BZE

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 11.58%, Coeff. Det.(r2): 0.999901
 $y = +1.3000x - 0.0199$



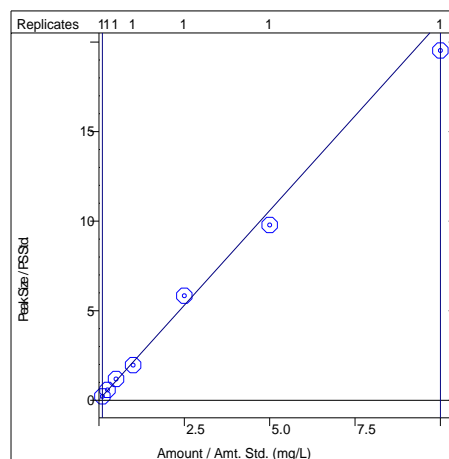
Cocaethylene

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 12.08%, Coeff. Det.(r2): 0.995783
 $y = +0.8788x - 0.0155$



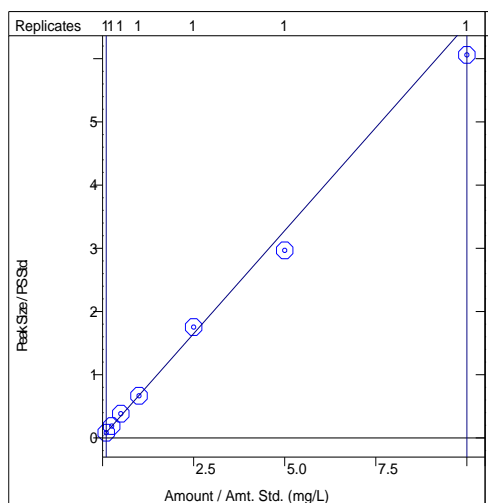
Codeine

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 9.170%, Coeff. Det.(r2): 0.997637
 $y = +2.1143x + 0.0202$



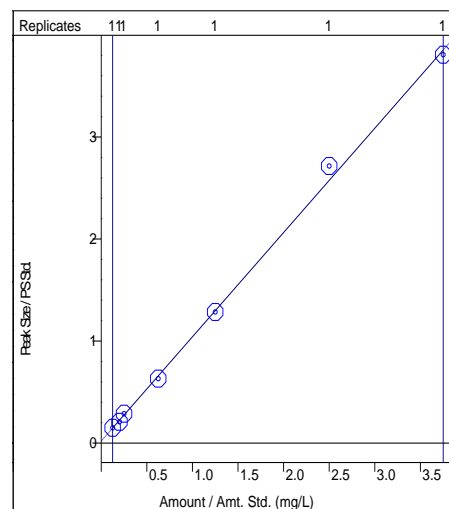
Morphine

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 12.54%, Coeff. Det.(r2): 0.998321
 $y = +0.6518x + 0.0205$



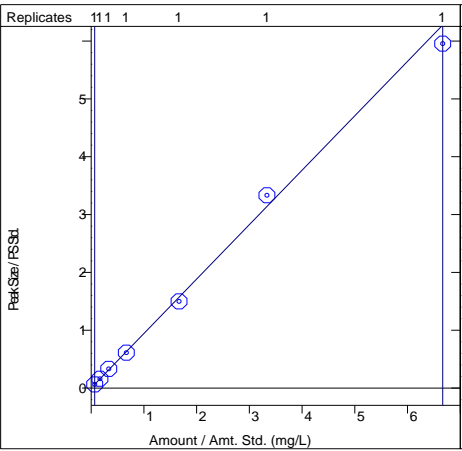
6MAM

Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
 Resp. Fact. RSD: 6.936%, Coeff. Det.(r2): 0.998180
 $y = +1.0221x + 0.0194$

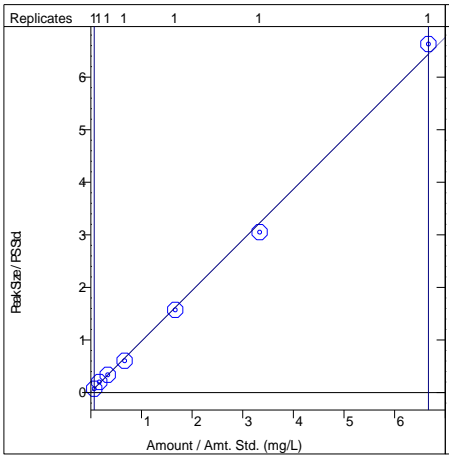


Appendix 12 Muscle validation: Calibration curves

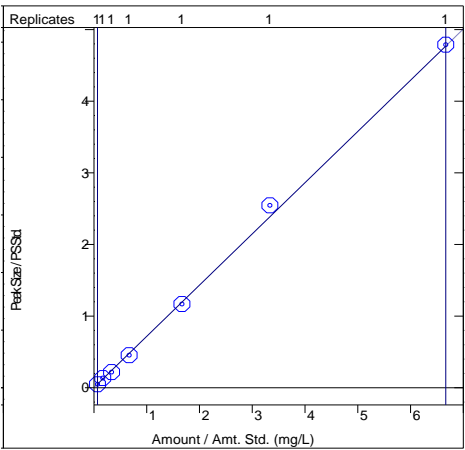
Cocaine
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 4.771%, Coeff. Det.(r2): 0.996666
 $y = +0.9411x + 0.0022$



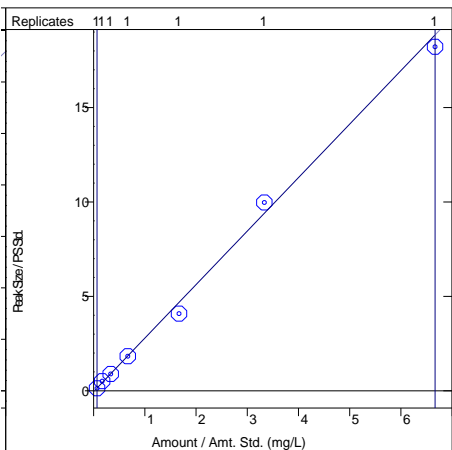
BZE
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 10.80%, Coeff. Det.(r2): 0.998287
 $y = +0.9637x + 0.0128$



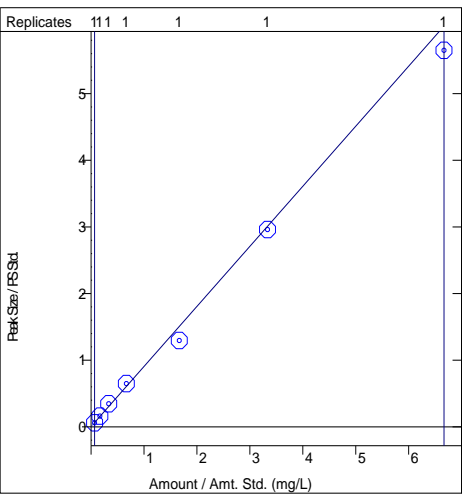
Cocaethylene
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 7.314%, Coeff. Det.(r2): 0.998814
 $y = +0.7150x + 0.0029$



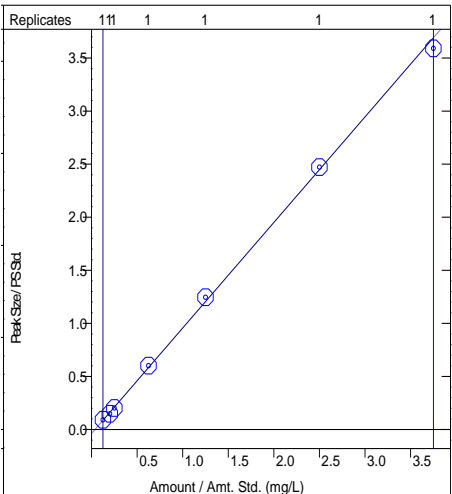
Codeine
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 13.30%, Coeff. Det.(r2): 0.996707
 $y = +2.8332x - 0.0426$



Morphine
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 9.677%, Coeff. Det.(r2): 0.998486
 $y = +0.9012x + 0.0047$



6MAM
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 13.31%, Coeff. Det.(r2): 0.999213
 $y = +0.9944x - 0.0381$



Appendix 13 Aqueous validation data (mg/L): Repeatability, accuracy, intermediate precision

		COC	COET	BZE	COD	MOR	6AM
	Nominal concentration (mg/L)	0.025	0.025	0.025	0.025	0.025	0.01
Exp 1	LOW 1.1	0.026	0.025	0.024	0.025	0.026	0.011
	LOW 1.2	0.027	0.025	0.025	0.025	0.025	0.011
	LOW 1.3	0.024	0.028	0.027	0.022	0.025	0.009
	LOW 1.4	0.028	0.03	0.025	0.023	0.026	0.01
	LOW 1.5	0.026	0.03	0.022	0.022	0.024	0.009
	LOW 1.6	0.027	0.029	0.027	0.023	0.024	0.01
	<i>Mean</i>	<i>0.026</i>	<i>0.028</i>	<i>0.025</i>	<i>0.023</i>	<i>0.025</i>	<i>0.010</i>
	<i>Standard deviation</i>	<i>0.001</i>	<i>0.003</i>	<i>0.002</i>	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>
	<i>Repeatability (% RSD)</i>	<i>5</i>	<i>9</i>	<i>7</i>	<i>5</i>	<i>4</i>	<i>9</i>
	<i>Accuracy (% deviation)</i>	<i>-5</i>	<i>-11</i>	<i>0</i>	<i>8</i>	<i>0</i>	<i>0</i>
Exp 2	LOW 2.1	0.024	0.027	0.027	0.026	0.027	0.01
	LOW 2.2	0.026	0.025	0.025	0.025	0.029	0.011
	LOW 2.3	0.024	0.025	0.022	0.027	0.029	0.011
Exp 3	LOW 3.1	0.025	0.023	0.022	0.024	0.023	0.011
	LOW 3.2	0.023	0.023	0.026	0.021	0.022	0.009
	LOW 3.3	0.025	0.022	0.025	0.025	0.026	0.010
	<i>Mean</i>	<i>0.025</i>	<i>0.026</i>	<i>0.025</i>	<i>0.024</i>	<i>0.025</i>	<i>0.010</i>
	<i>Standard deviation</i>	<i>0.001</i>	<i>0.003</i>	<i>0.002</i>	<i>0.002</i>	<i>0.002</i>	<i>0.001</i>
	<i>Intermediate precision (% RSD)</i>	<i>6</i>	<i>11</i>	<i>8</i>	<i>8</i>	<i>9</i>	<i>9</i>
	Nominal concentration (mg/L)	0.15	0.15	0.15	0.15	0.15	0.05
Exp 1	MEDIUM 1.1	0.14	0.15	0.15	0.13	0.15	0.048
	MEDIUM 1.2	0.15	0.15	0.12	0.15	0.16	0.046
	MEDIUM 1.3	0.16	0.16	0.13	0.13	0.16	0.04
	MEDIUM 1.4	0.14	0.16	0.13	0.13	0.15	0.055
	MEDIUM 1.5	0.14	0.13	0.13	0.13	0.14	0.051
	MEDIUM 1.6	0.16	0.13	0.15	0.14	0.15	0.048
	<i>Mean</i>	<i>0.15</i>	<i>0.15</i>	<i>0.14</i>	<i>0.14</i>	<i>0.15</i>	<i>0.048</i>
	<i>Standard deviation</i>	<i>0.008</i>	<i>0.014</i>	<i>0.013</i>	<i>0.007</i>	<i>0.008</i>	<i>0.005</i>
	<i>Repeatability (% RSD)</i>	<i>5</i>	<i>10</i>	<i>10</i>	<i>5</i>	<i>6</i>	<i>10</i>
	<i>Accuracy (% deviation)</i>	<i>1</i>	<i>1</i>	<i>9</i>	<i>10</i>	<i>0</i>	<i>4</i>
Exp 2	MEDIUM 2.1	0.16	0.12	0.18	0.17	0.17	0.05
	MEDIUM 2.2	0.14	0.14	0.18	0.17	0.17	0.059
	MEDIUM 2.3	0.13	0.14	0.18	0.17	0.17	0.055
Exp 3	MEDIUM 3.1	0.15	0.14	0.12	0.12	0.13	0.044
	MEDIUM 3.2	0.14	0.17	0.12	0.17	0.13	0.044
	MEDIUM 3.3	0.16	0.17	0.12	0.16	0.14	0.051
	<i>Mean</i>	<i>0.15</i>	<i>0.15</i>	<i>0.14</i>	<i>0.15</i>	<i>0.15</i>	<i>0.049</i>
	<i>Standard deviation</i>	<i>0.010</i>	<i>0.016</i>	<i>0.024</i>	<i>0.019</i>	<i>0.015</i>	<i>0.005</i>
	<i>Intermediate precision (% RSD)</i>	<i>7</i>	<i>11</i>	<i>17</i>	<i>13</i>	<i>10</i>	<i>11</i>
	Nominal concentration (mg/L)	0.4	0.4	0.4	0.4	0.4	0.1
Exp 1	HIGH 1.1	0.43	0.38	0.38	0.37	0.34	0.11
	HIGH 1.2	0.42	0.39	0.41	0.38	0.35	0.096
	HIGH 1.3	0.46	0.46	0.35	0.38	0.38	0.099
	HIGH 1.4	0.46	0.46	0.37	0.34	0.39	0.093

	HIGH 1.5	0.45	0.43	0.34	0.34	0.37	0.091
	HIGH 1.6	0.45	0.44	0.35	0.35	0.35	0.089
	<i>Mean</i>	<i>0.44</i>	<i>0.43</i>	<i>0.37</i>	<i>0.36</i>	<i>0.36</i>	<i>0.096</i>
	<i>Standard deviation</i>	<i>0.017</i>	<i>0.034</i>	<i>0.024</i>	<i>0.018</i>	<i>0.020</i>	<i>0.008</i>
	<i>Repeatability (% RSD)</i>	<i>4</i>	<i>8</i>	<i>6</i>	<i>5</i>	<i>5</i>	<i>8</i>
	<i>Accuracy (% deviation)</i>	<i>-11</i>	<i>-7</i>	<i>7</i>	<i>10</i>	<i>9</i>	<i>4</i>
Exp 2	HIGH 2.1	0.32	0.45	0.42	0.44	0.40	0.10
	HIGH 2.2	0.33	0.40	0.47	0.42	0.39	0.10
	HIGH 2.3	0.35	0.46	0.46	0.40	0.38	0.099
Exp 3	HIGH 3.1	0.48	0.39	0.37	0.43	0.35	0.094
	HIGH 3.2	0.48	0.40	0.41	0.42	0.35	0.099
	HIGH 3.3	0.47	0.40	0.36	0.45	0.39	0.096
	<i>Mean</i>	<i>0.42</i>	<i>0.42</i>	<i>0.39</i>	<i>0.39</i>	<i>0.37</i>	<i>0.097</i>
	<i>SD</i>	<i>0.058</i>	<i>0.032</i>	<i>0.040</i>	<i>0.038</i>	<i>0.021</i>	<i>0.005</i>
	<i>Intermediate precision (% RSD)</i>	<i>14</i>	<i>8</i>	<i>10</i>	<i>10</i>	<i>6</i>	<i>6</i>

Appendix 14 Blood validation data (mg/L): Repeatability, accuracy, intermediate precision

		COC	COET	BZE	COD	MOR	6AM
	Nominal concentration (mg/L)	0.025	0.025	0.025	0.025	0.025	0.01
Exp 1	LOW 1.1	0.027	0.025	0.027	0.021	0.024	0.01
	LOW 1.2	0.025	0.024	0.025	0.02	0.021	0.009
	LOW 1.3	0.025	0.022	0.022	0.02	0.018	0.009
	LOW 1.4	0.024	0.025	0.025	0.022	0.023	0.008
	LOW 1.5	0.024	0.023	0.021	0.025	0.028	0.008
	LOW 1.6	0.023	0.028	0.024	0.027	0.028	0.008
	<i>Mean</i>	0.025	0.025	0.024	0.023	0.024	0.009
	<i>Standard deviation</i>	0.001	0.002	0.002	0.003	0.004	0.001
	<i>Repeatability (% RSD)</i>	6	8	9	13	17	9
	<i>Accuracy (% deviation)</i>	1	2	4	10	5	13
Exp 2	LOW 2.1	0.022	0.027	0.026	0.021	0.024	0.011
	LOW 2.2	0.024	0.025	0.023	0.024	0.021	0.01
	LOW 2.3	0.023	0.026	0.02	0.026	0.023	0.011
Exp 3	LOW 3.1	0.027	0.026	0.023	0.028	0.027	0.011
	LOW 3.2	0.024	0.028	0.024	0.028	0.028	0.010
	LOW 3.3	0.026	0.029	0.022	0.026	0.028	0.012
	<i>Mean</i>	0.024	0.026	0.023	0.024	0.024	0.010
	<i>Standard deviation</i>	0.002	0.002	0.002	0.003	0.003	0.001
	<i>Intermediate precision (% RSD)</i>	6	8	9	13	13	14
	Nominal concentration (mg/L)	0.15	0.15	0.15	0.15	0.15	0.05
Exp 1	MEDIUM 1.1	0.13	0.14	0.14	0.15	0.17	0.054
	MEDIUM 1.2	0.16	0.16	0.13	0.12	0.16	0.055
	MEDIUM 1.3	0.15	0.14	0.14	0.16	0.17	0.066
	MEDIUM 1.4	0.15	0.14	0.13	0.13	0.17	0.057
	MEDIUM 1.5	0.16	0.15	0.13	0.15	0.18	0.059
	MEDIUM 1.6	0.14	0.14	0.14	0.15	0.19	0.056
	<i>Mean</i>	0.15	0.14	0.14	0.14	0.17	0.058
	<i>Standard deviation</i>	0.010	0.008	0.006	0.015	0.012	0.004
	<i>Repeatability (% RSD)</i>	7	6	5	10	7	8
	<i>Accuracy (% deviation)</i>	0.2	5	10	5	-15	-16
Exp 2	MEDIUM 2.1	0.16	0.16	0.16	0.20	0.16	0.055
	MEDIUM 2.2	0.17	0.18	0.17	0.15	0.18	0.052
	MEDIUM 2.3	0.17	0.18	0.18	0.16	0.16	0.058
Exp 3	MEDIUM 3.1	0.16	0.16	0.17	0.16	0.16	0.050
	MEDIUM 3.2	0.17	0.17	0.18	0.18	0.14	0.050
	MEDIUM 3.3	0.17	0.17	0.16	0.15	0.17	0.058
	<i>Mean</i>	0.16	0.16	0.15	0.15	0.17	0.056
	<i>Standard deviation</i>	0.012	0.016	0.019	0.019	0.013	0.004
	<i>Intermediate precision (% RSD)</i>	7	10	13	12	8	8
	Nominal concentration (mg/L)	0.4	0.4	0.4	0.4	0.4	0.1
Exp 1	HIGH 1.1	0.44	0.44	0.41	0.31	0.39	0.14
	HIGH 1.2	0.47	0.45	0.39	0.41	0.48	0.11
	HIGH 1.3	0.39	0.40	0.38	0.41	0.49	0.099
	HIGH 1.4	0.40	0.41	0.40	0.40	0.48	0.10
	HIGH 1.5	0.40	0.38	0.42	0.36	0.41	0.12
	HIGH 1.6	0.41	0.42	0.43	0.38	0.42	0.12
	<i>Mean</i>	0.42	0.42	0.40	0.38	0.45	0.12
	<i>Standard deviation</i>	0.030	0.025	0.018	0.038	0.043	0.015
	<i>Repeatability (% RSD)</i>	7	6	5	10	10	13
	<i>Accuracy (% deviation)</i>	-4	-4	-1	6	-11	-15
Exp 2	HIGH 2.1	0.47	0.45	0.47	0.42	0.40	0.099
	HIGH 2.2	0.46	0.40	0.47	0.46	0.42	0.10

Exp 3	HIGH 2.3	0.42	0.44	0.46	0.44	0.39	0.11
	HIGH 3.1	0.44	0.45	0.46	0.45	0.37	0.11
	HIGH 3.2	0.43	0.44	0.47	0.43	0.39	0.097
	HIGH 3.3	0.45	0.45	0.45	0.48	0.39	0.11
<i>Mean</i>		<i>0.43</i>	<i>0.43</i>	<i>0.43</i>	<i>0.41</i>	<i>0.42</i>	<i>0.11</i>
<i>Standard deviation</i>		<i>0.028</i>	<i>0.024</i>	<i>0.034</i>	<i>0.045</i>	<i>0.041</i>	<i>0.012</i>
<i>Intermediate precision (% RSD)</i>		<i>6</i>	<i>6</i>	<i>8</i>	<i>11</i>	<i>10</i>	<i>11</i>

Appendix 15 Muscle validation data (mg/0.5kg): Repeatability, accuracy, intermediate precision

		COC	COET	BZE	COD	MOR	6AM
Nominal concentration (mg/0.5kg)		0.025	0.025	0.025	0.025	0.025	0.01
Exp 1	LOW 1.1	0.02	0.027	0.031	0.024	0.037	0.013
	LOW 1.2	0.024	0.024	0.028	0.023	0.028	0.009
	LOW 1.3	0.02	0.026	0.028	0.023	0.03	0.011
	LOW 1.4	0.023	0.027	0.03	0.028	0.027	0.012
	LOW 1.5	0.021	0.024	0.029	0.023	0.022	0.011
	LOW 1.6	0.02	0.023	0.028	0.03	0.029	0.01
<i>Mean</i>		<i>0.021</i>	<i>0.025</i>	<i>0.029</i>	<i>0.025</i>	<i>0.029</i>	<i>0.011</i>
<i>Standard deviation</i>		<i>0.002</i>	<i>0.002</i>	<i>0.001</i>	<i>0.003</i>	<i>0.005</i>	<i>0.001</i>
<i>Repeatability (% RSD)</i>		<i>8</i>	<i>7</i>	<i>4</i>	<i>12</i>	<i>17</i>	<i>13</i>
<i>Accuracy (% deviation)</i>		<i>15</i>	<i>-1</i>	<i>-16</i>	<i>-1</i>	<i>-15</i>	<i>-10</i>
Exp 2	LOW 2.1	0.028	0.026	0.03	0.032	0.029	0.01
	LOW 2.2	0.027	0.026	0.027	0.029	0.026	0.012
	LOW 2.3	0.025	0.023	0.023	0.026	0.03	0.01
Exp 3	LOW 3.1	0.025	0.024	0.03	0.023	0.029	0.01
	LOW 3.2	0.027	0.027	0.028	0.022	0.026	0.012
	LOW 3.3	0.026	0.026	0.021	0.021	0.027	0.011
<i>Mean</i>		<i>0.024</i>	<i>0.025</i>	<i>0.028</i>	<i>0.025</i>	<i>0.028</i>	<i>0.011</i>
<i>Standard deviation</i>		<i>0.003</i>	<i>0.002</i>	<i>0.003</i>	<i>0.004</i>	<i>0.004</i>	<i>0.001</i>
<i>Intermediate precision (% RSD)</i>		<i>13</i>	<i>6</i>	<i>11</i>	<i>14</i>	<i>12</i>	<i>11</i>
Nominal concentration (mg/0.5kg)		0.15	0.15	0.15	0.15	0.15	0.05
Exp 1	MEDIUM 1.1	0.14	0.14	0.14	0.13	0.18	0.062
	MEDIUM 1.2	0.17	0.15	0.14	0.11	0.14	0.063
	MEDIUM 1.3	0.16	0.15	0.14	0.12	0.15	0.055
	MEDIUM 1.4	0.16	0.14	0.14	0.12	0.16	0.06
	MEDIUM 1.5	0.15	0.14	0.13	0.13	0.18	0.062
	MEDIUM 1.6	0.16	0.15	0.14	0.13	0.15	0.051
<i>Mean</i>		<i>0.16</i>	<i>0.15</i>	<i>0.14</i>	<i>0.12</i>	<i>0.16</i>	<i>0.059</i>
<i>Standard deviation</i>		<i>0.009</i>	<i>0.005</i>	<i>0.007</i>	<i>0.007</i>	<i>0.017</i>	<i>0.005</i>
<i>Repeatability (% RSD)</i>		<i>6</i>	<i>3</i>	<i>5</i>	<i>6</i>	<i>11</i>	<i>8</i>
<i>Accuracy (% deviation)</i>		<i>-3</i>	<i>3</i>	<i>7</i>	<i>19</i>	<i>-6</i>	<i>-18</i>
Exp 2	MEDIUM 2.1	0.16	0.17	0.16	0.13	0.17	0.043
	MEDIUM 2.2	0.18	0.19	0.17	0.18	0.19	0.055
	MEDIUM 2.3	0.16	0.17	0.16	0.14	0.18	0.07
Exp 3	MEDIUM 3.1	0.14	0.15	0.14	0.14	0.15	0.057
	MEDIUM 3.2	0.14	0.14	0.13	0.13	0.14	0.05
	MEDIUM 3.3	0.14	0.14	0.14	0.17	0.14	0.055
<i>Mean</i>		<i>0.15</i>	<i>0.15</i>	<i>0.14</i>	<i>0.13</i>	<i>0.16</i>	<i>0.057</i>
<i>Standard deviation</i>		<i>0.013</i>	<i>0.015</i>	<i>0.013</i>	<i>0.018</i>	<i>0.018</i>	<i>0.007</i>
<i>Intermediate precision (% RSD)</i>		<i>8</i>	<i>10</i>	<i>9</i>	<i>14</i>	<i>11</i>	<i>13</i>
Nominal concentration (mg/0.5kg)		0.4	0.4	0.4	0.4	0.4	0.1
Exp 1	HIGH 1.1	0.38	0.39	0.42	0.31	0.41	0.10
	HIGH 1.2	0.41	0.44	0.40	0.36	0.45	0.11
	HIGH 1.3	0.48	0.48	0.41	0.32	0.47	0.12
	HIGH 1.4	0.42	0.41	0.42	0.34	0.41	0.12
	HIGH 1.5	0.46	0.48	0.45	0.36	0.38	0.12
	HIGH 1.6	0.43	0.43	0.42	0.34	0.37	0.11
<i>Mean</i>		<i>0.43</i>	<i>0.44</i>	<i>0.42</i>	<i>0.34</i>	<i>0.41</i>	<i>0.11</i>
<i>Standard deviation</i>		<i>0.036</i>	<i>0.034</i>	<i>0.016</i>	<i>0.019</i>	<i>0.037</i>	<i>0.008</i>
<i>Repeatability (% RSD)</i>		<i>8</i>	<i>8</i>	<i>4</i>	<i>6</i>	<i>9</i>	<i>7</i>

	<i>Accuracy (% deviation)</i>	<i>-8</i>	<i>-9</i>	<i>-5</i>	<i>16</i>	<i>-3</i>	<i>-13</i>
Exp 2	HIGH 2.1	0.38	0.39	0.35	0.33	0.31	0.084
	HIGH 2.2	0.37	0.38	0.36	0.41	0.32	0.091
	HIGH 2.3	0.38	0.36	0.37	0.32	0.32	0.10
Exp 3	HIGH 3.1	0.37	0.38	0.33	0.36	0.32	0.098
	HIGH 3.2	0.35	0.34	0.35	0.39	0.35	0.11
	HIGH 3.3	0.36	0.38	0.33	0.38	0.32	0.099
<i>Mean</i>		<i>0.40</i>	<i>0.41</i>	<i>0.38</i>	<i>0.35</i>	<i>0.37</i>	<i>0.11</i>
<i>SD</i>		<i>0.041</i>	<i>0.043</i>	<i>0.040</i>	<i>0.030</i>	<i>0.054</i>	<i>0.011</i>
<i>Intermediate precision (% RSD)</i>		<i>10</i>	<i>11</i>	<i>10</i>	<i>9</i>	<i>15</i>	<i>11</i>

Appendix 16 Aqueous validation data (mg/L): Limit of quantification

	COC	COET	BZE	COD	MOR	6AM
Nominal concentration (ng/mL)	10	10	10	10	10	5
LOQ 1.1	12	11	10	10	12	5.8
LOQ 1.2	9.6	12	11	9.7	10	6.3
LOQ 1.3	9.8	11	10	9.0	11	4.7
LOQ 1.4	10	12	11	7.9	11	4.1
LOQ 1.5	9.7	11	12	9.2	9.7	4.5
LOQ 1.6	10	12	12	8.7	9.6	3.8
<i>Mean</i>	<i>10.2</i>	<i>11.3</i>	<i>11.0</i>	<i>9.1</i>	<i>10.6</i>	<i>4.9</i>
<i>Standard deviation</i>	<i>0.7</i>	<i>0.5</i>	<i>0.9</i>	<i>0.8</i>	<i>1.0</i>	<i>1.0</i>
<i>Repeatability (% RSD)</i>	<i>7</i>	<i>4</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>20</i>
<i>Accuracy (% deviation)</i>	<i>-2</i>	<i>-13</i>	<i>-10</i>	<i>9</i>	<i>-6</i>	<i>2</i>
Nominal concentration (ng/mL)	7.5	7.5	7.5	7.5	7.5	4
LOQ 2.1	7.3	8.2	7.1	5.8	8.4	3.2
LOQ 2.2	6.8	7.8	7.6	7.5	8.4	3.2
LOQ 2.3	7.3	8.0	8.1	7.0	9.1	3.7
LOQ 2.4	7.0	7.6	6.6	7.0	8.6	3.2
LOQ 2.5	7.5	7.8	8.1	6.9	7.6	4.3
LOQ 2.6	7.5	8.8	6.7	6.6	7.7	5.0
<i>Mean</i>	<i>7.2</i>	<i>8.0</i>	<i>7.4</i>	<i>6.8</i>	<i>8.3</i>	<i>3.8</i>
<i>SD</i>	<i>0.3</i>	<i>0.4</i>	<i>0.7</i>	<i>0.6</i>	<i>0.6</i>	<i>0.7</i>
<i>Precision</i>	<i>4</i>	<i>5</i>	<i>9</i>	<i>8</i>	<i>7</i>	<i>20</i>
<i>Accuracy</i>	<i>3</i>	<i>-7</i>	<i>2</i>	<i>9</i>	<i>-11</i>	<i>6</i>
Nominal concentration (ng/mL)	5	5	5	5	5	3
LOQ 3.1	4.6	5.0	4.0	4.2	6.0	2.6
LOQ 3.2	4.7	5.3	4.9	4.3	5.9	2.5
LOQ 3.3	4.8	4.9	5.2	4.3	5.9	2.2
LOQ 3.4	5.2	5.8	5.4	4.3	5.9	2.4
LOQ 3.5	4.2	4.9	5.0	4.7	5.9	3.8
LOQ 3.6	4.3	4.6	5.7	5.0	5.9	3.0
<i>Mean</i>	<i>4.6</i>	<i>5.1</i>	<i>5.0</i>	<i>4.5</i>	<i>5.9</i>	<i>2.8</i>
<i>Standard deviation</i>	<i>0.4</i>	<i>0.4</i>	<i>0.6</i>	<i>0.3</i>	<i>0.1</i>	<i>0.6</i>
<i>Repeatability (% RSD)</i>	<i>8</i>	<i>9</i>	<i>12</i>	<i>7</i>	<i>1</i>	<i>22</i>
<i>Accuracy (% deviation)</i>	<i>7</i>	<i>-2</i>	<i>-1</i>	<i>11</i>	<i>-18</i>	<i>8</i>
Nominal concentration (ng/mL)	2.5	2.5	2.5	2.5	2.5	2
LOQ 4.1	2.7	3.0	2.6	2.5	3.9	1.8
LOQ 4.2	2.6	3.1	3.0	2.7	4.0	1.5
LOQ 4.2	2.8	3.0	2.5	2.6	4.0	1.4
LOQ 4.3	2.8	3.1	2.7	2.8	3.8	1.7
LOQ 4.3	2.8	2.8	2.6	2.4	3.8	1.6
LOQ 4.3	2.8	3.0	3	2.9	4.1	1.8
<i>Mean</i>	<i>2.8</i>	<i>3.0</i>	<i>2.7</i>	<i>2.6</i>	<i>3.9</i>	<i>1.6</i>
<i>Standard deviation</i>	<i>0.1</i>	<i>0.1</i>	<i>0.2</i>	<i>0.2</i>	<i>0.1</i>	<i>0.2</i>
<i>Repeatability (% RSD)</i>	<i>3</i>	<i>4</i>	<i>8</i>	<i>7</i>	<i>3</i>	<i>10</i>
<i>Accuracy (% deviation)</i>	<i>-10</i>	<i>-20</i>	<i>-10</i>	<i>-5</i>	<i>-57</i>	<i>19</i>
Nominal concentration (ng/mL)	1	1	1	1	1	1
LOQ 5.1	1.6	2.4	ND	1.5	ND	ND
LOQ 5.2	ND	ND	1.5	1.4	ND	ND
LOQ 5.3	2.0	2.3	2.0	1.4	ND	ND
LOQ 5.4	2.0	3.0	2.0	1.8	ND	ND
LOQ 5.5	2.2	3.3	ND	2.0	ND	ND
LOQ 5.6	2.3	2.9	2.5	2.0	ND	ND
<i>Mean</i>	<i>2.0</i>	<i>2.8</i>	<i>2.0</i>	<i>1.7</i>		
<i>Standard deviation</i>	<i>0.3</i>	<i>0.4</i>	<i>0.4</i>	<i>0.3</i>		
<i>Repeatability (% RSD)</i>	<i>13</i>	<i>15</i>	<i>21</i>	<i>17</i>		
<i>Accuracy (% deviation)</i>	<i>-103</i>	<i>-177</i>	<i>-99</i>	<i>-70</i>		

Appendix 17 Blood validation data (mg/L): Limit of quantification

	COC	COET	BZE	COD	MOR	6AM
Nominal concentration (ng/mL)	10	10	10	10	10	5
LOQ 1.1	9.4	8.8	10.3	8.7	9.6	5.1
LOQ 1.2	8.6	9.5	10.5	9.1	9.8	5.1
LOQ 1.3	8.8	8.2	11.2	8.4	8.9	5.0
LOQ 1.4	9.0	8.8	8.7	8.6	9.8	4.6
LOQ 1.5	8.4	9.4	10.4	8.5	9.1	5.2
LOQ 1.6	8.1	7.7	8.5	8.1	8.0	4.6
<i>Mean</i>	8.7	8.7	9.9	8.6	9.2	4.9
<i>Standard deviation</i>	0.5	0.7	1.1	0.3	0.7	0.3
<i>Repeatability (% RSD)</i>	5	8	11	4	8	5
<i>Accuracy (% deviation)</i>	13	13	1	14	8	1
Nominal concentration (ng/mL)	7.5	7.5	7.5	7.5	7.5	4
LOQ 2.1	8.5	7.3	9.3	7.9	7.1	4.0
LOQ 2.2	8.6	6.7	8.1	7.4	6.7	4.4
LOQ 2.3	7.8	7.5	7.2	7.3	6.6	3.8
LOQ 2.4	6.1	6.7	7.3	7.6	6.7	3.9
LOQ 2.5	6.2	6.8	7.6	7.7	6.7	4.1
LOQ 2.6	6.0	6.6	8.3	7.6	6.4	4.6
<i>Mean</i>	7.2	6.9	8.0	7.6	6.7	4.1
<i>SD</i>	1.2	0.4	0.8	0.2	0.2	0.3
<i>Precision</i>	17	6	10	3	3	7
<i>Accuracy</i>	4	8	-6	-1	11	-3
Nominal concentration (ng/mL)	5	5	5	5	5	3
LOQ 3.1	4.1	4.5	5.5	6.0	5.4	2.6
LOQ 3.2	5.3	4.1	4.7	5.5	5.8	2.7
LOQ 3.3	4.4	4.6	6.1	5.0	5.8	3.3
LOQ 3.4	5.2	4.4	4.4	5.4	5.8	2.4
LOQ 3.5	5.4	5.0	4.8	6.0	5.7	3.2
LOQ 3.6	5.2	4.4	5.0	5.5	5.9	2.6
<i>Mean</i>	4.9	4.5	5.1	5.6	5.7	2.8
<i>Standard deviation</i>	0.5	0.3	0.6	0.4	0.2	0.4
<i>Repeatability (% RSD)</i>	11	7	12	7	3	13
<i>Accuracy (% deviation)</i>	1	10	-1	-11	-15	7
Nominal concentration (ng/mL)	2.5	2.5	2.5	2.5	2.5	2
LOQ 4.1	1.2	1.9	1.0	5.4	4.9	1.5
LOQ 4.2	1.8	2.0	1.4	5.4	4.9	1.1
LOQ 4.2	2.2	3.6	1.6	5.5	4.9	1.4
LOQ 4.3	2.5	2.7	1.4	5.4	4.9	0.92
LOQ 4.3	1.6	2.3	0.48	5.7	4.8	1.4
LOQ 4.3	1.3	2.4	0.36	5.3	4.9	1.5
<i>Mean</i>	1.8	2.5	1.0	5.5	4.9	1.3
<i>Standard deviation</i>	0.5	0.6	0.5	0.1	0.0	0.3
<i>Repeatability (% RSD)</i>	29	25	50	2	1	19
<i>Accuracy (% deviation)</i>	30	1	59	-5	-95	34
Nominal concentration (ng/mL)	1	1	1	1	1	1
LOQ 5.1	0.30	0.88	1.1	4.8	ND	ND
LOQ 5.2	0.055	0.94	0.65	4.8	ND	0.10
LOQ 5.3	0.091	0.85	0.29	4.9	ND	ND
LOQ 5.4	0.49	1.2	0.093	5.2	4.7	0.66
LOQ 5.5	0.092	0.9	0.085	4.9	ND	0.26
LOQ 5.6	0.44	0.81	0.024	4.8	ND	0.23
<i>Mean</i>	0.2	0.9	0.4	4.9	4.7	0.3
<i>Standard deviation</i>	0.2	0.1	0.4	0.1		0.2
<i>Repeatability (% RSD)</i>	78	14	114	3		78
<i>Accuracy (% deviation)</i>	76	8	62	-392	-368	69

Appendix 18 Muscle validation data (mg/0.5kg): Limit of quantification

	COC	COET	BZE	COD	MOR	6AM
Nominal concentration (ng/0.5g)	10	10	10	10	10	5
LOQ 1.1	8.4	9.2	12	9.5	12	4.7
LOQ 1.2	11	8.2	9.6	8.3	9.2	5.4
LOQ 1.3	11	8.7	9.9	8.5	11	5.1
LOQ 1.4	11	11	9.7	10	12	4.9
LOQ 1.5	8	8.6	11	9.2	12	5.1
LOQ 1.6	9	9.7	8.1	9.7	12	5
<i>Mean</i>	9.6	9.3	10	9.2	11	5.0
<i>Standard deviation</i>	1.3	1.2	1.2	0.7	1.0	0.2
<i>Repeatability (% RSD)</i>	13	12	12	7	9	5
<i>Accuracy (% deviation)</i>	4	7	1	8	-11	-1
Nominal concentration (ng/0.5g)	7.5	7.5	7.5	7.5	7.5	4
LOQ 2.1	7.4	8.1	7.6	9.5	8	4.8
LOQ 2.2	8.6	8.4	9.4	8.5	7.6	3.7
LOQ 2.3	9	8.9	9.3	8.6	8.3	4.8
LOQ 2.4	8.9	7	7.1	7.4	9.4	4
LOQ 2.5	7.7	6.4	9	8.2	9	4.3
LOQ 2.6	8.3	6.9	8.8	8.5	8	3.4
<i>Mean</i>	8.3	7.6	8.5	8.5	8.4	4.2
<i>SD</i>	0.6	1.0	1.0	0.7	0.7	0.6
<i>Precision</i>	8	13	11	8	8	14
<i>Accuracy</i>	-11	-2	-14	-13	-12	-4
Nominal concentration (ng/0.5g)	5	5	5	5	5	3
LOQ 3.1	5.2	5.1	4.7	5.9	5.6	2.6
LOQ 3.2	5.7	4.5	5	6.4	5.4	3.6
LOQ 3.3	5.4	5.7	5	4.8	5.1	3.6
LOQ 3.4	5.1	5.9	6.4	5.2	5.6	3
LOQ 3.5	5.3	5.3	5.7	5.9	4.9	2.7
LOQ 3.6	5.2	5.4	6.2	6.2	6	2.5
<i>Mean</i>	5.3	5.3	5.5	5.7	5.4	3.0
<i>Standard deviation</i>	0.2	0.5	0.7	0.6	0.4	0.5
<i>Repeatability (% RSD)</i>	4	9	13	11	7	16
<i>Accuracy (% deviation)</i>	-6	-6	-10	-15	-9	0
Nominal concentration (ng/0.5g)	2.5	2.5	2.5	2.5	2.5	2
LOQ 4.1	3.4	3	0	2.7	3.7	2.6
LOQ 4.2	3.4	2.9	4.9	2.5	3.4	1.6
LOQ 4.2	2.5	3.2	5	3.2	2.9	1.3
LOQ 4.3	2.4	0	0	3.1	2.8	1.6
LOQ 4.3	2.9	2.6	2.3	0.7	3	2.6
LOQ 4.3	2.6	2.7	0	2.6	3.3	1.6
<i>Mean</i>	2.9	2.4	2.0	2.5	3.2	1.9
<i>Standard deviation</i>	0.4	1.2	2.4	0.9	0.3	0.6
<i>Repeatability (% RSD)</i>	16	50	119	37	11	30
<i>Accuracy (% deviation)</i>	-15	4	19	-5	-27	6
Nominal concentration (ng/0.5g)	1	1	1	1	1	1
LOQ 5.1	0	0	0	0.1	1.3	1.1
LOQ 5.2	2	0	0	0.6	1.6	0.3
LOQ 5.3	1.8	0	0	0.7	1.8	0.1
LOQ 5.4	3.9	0	1.6	0.3	2	0.6
LOQ 5.5	4	1.3	1.1	0.5	0	1.3
LOQ 5.6	3.1	0	1.4	0	1.2	0.7
<i>Mean</i>	2.5	0.2	0.7	0.4	1.3	0.7
<i>Standard deviation</i>	1.5	0.5	0.8	0.3	0.7	0.5
<i>Repeatability (% RSD)</i>	62	245	112	76	54	67
<i>Accuracy (% deviation)</i>	-147	78	32	63	-32	32

Appendix 19 Total morphine validation data (mg/L): hydrolytic efficiency and precision in blood

	Sample ID	Free morphine (mg/L)	Efficiency %
	Nominal	0.10	100%
Exp 1	LQC 1.1	0.086	86
	LQC 1.2	0.11	105
	LQC 1.3	0.091	91
	LQC 1.4	0.094	94
	LQC 1.5	0.077	77
	LQC 1.6	NR	
	<i>Mean</i>	<i>0.091</i>	<i>91</i>
	<i>Standard deviation</i>	<i>0.010</i>	<i>10</i>
	<i>Repeatability (% RSD)</i>	<i>11</i>	<i>11</i>
Exp 2	LQC 2.1	0.086	86
	LQC 2.2	0.09	90
	LQC 2.3	0.095	95
	LQC 2.4	0.073	73
	LQC 2.5	0.09	90
	LQC 2.6	0.08	80
	<i>Mean</i>	<i>0.088</i>	<i>88</i>
	<i>Standard deviation</i>	<i>0.009</i>	<i>9</i>
	<i>Intermediate precision (% RSD)</i>	<i>10</i>	<i>10</i>
	Nominal	0.40	100%
Exp 1	HQC 1.1	0.38	94
	HQC 1.2	0.39	98
	HQC 1.3	0.37	93
	HQC 1.4	0.34	85
	HQC 1.5	0.34	86
	HQC 1.6	NR	
	<i>Mean</i>	<i>0.36</i>	<i>91</i>
	<i>Standard deviation</i>	<i>0.022</i>	<i>5</i>
	<i>Repeatability (% RSD)</i>	<i>6</i>	<i>6</i>
Exp 2	HQC 2.1	0.30	74
	HQC 2.2	0.34	86
	HQC 2.3	0.32	80
	HQC 2.4	0.36	89
	HQC 2.5	0.31	78
	HQC 2.6	0.30	75
	<i>Mean</i>	<i>0.34</i>	<i>85</i>
	<i>Standard deviation</i>	<i>0.031</i>	<i>8</i>
	<i>Intermediate precision (% RSD)</i>	<i>9</i>	<i>9</i>

Appendix 20 Total morphine validation data (mg/0.5kg): Hydrolytic efficiency and precision in muscle

	Sample ID	Free morphine (mg/L)	Efficiency %
	Nominal	0.10	100%
Exp 1	LQC 1.1	0.097	97
	LQC 1.2	0.086	86
	LQC 1.3	0.11	110
	LQC 1.4	0.09	90
	LQC 1.5	0.084	84
	LQC 1.6	NR	
	<i>Mean</i>	<i>0.093</i>	<i>93</i>
	<i>Standard deviation</i>	<i>0.011</i>	<i>11</i>
	<i>Repeatability (% RSD)</i>	<i>11</i>	<i>11</i>
Exp 2	LQC 2.1	0.081	81
	LQC 2.2	0.075	75
	LQC 2.3	0.11	110
	LQC 2.4	0.089	89
	LQC 2.5	0.10	101
	LQC 2.6	0.10	100
	<i>Mean</i>	<i>0.093</i>	<i>93</i>
	<i>Standard deviation</i>	<i>0.012</i>	<i>12</i>
	<i>Intermediate precision (% RSD)</i>	<i>12</i>	<i>12</i>
	Nominal	0.40	100%
Exp 1	HQC 1.1	0.37	92
	HQC 1.2	0.39	97
	HQC 1.3	0.4	100
	HQC 1.4	0.43	107
	HQC 1.5	0.41	102
	HQC 1.6	NR	
	<i>Mean</i>	<i>0.40</i>	<i>99</i>
	<i>Standard deviation</i>	<i>0.023</i>	<i>6</i>
	<i>Repeatability (% RSD)</i>	<i>6</i>	<i>6</i>
Exp 2	HQC 2.1	0.30	76
	HQC 2.2	0.34	86
	HQC 2.3	0.39	97
	HQC 2.4	0.35	87
	HQC 2.5	0.41	102
	HQC 2.6	0.30	76
	<i>Mean</i>	<i>0.37</i>	<i>93</i>
	<i>Standard deviation</i>	<i>0.043</i>	<i>11</i>
	<i>Intermediate precision (% RSD)</i>	<i>11</i>	<i>11</i>

NR: no result

Appendix 21 Stability Study: Cocaine (COC) and benzoylecgonine (BZE) concentration (mg/L) measured in triplicate blood samples over 84 days

	20 degrees C						4 degrees C						-18 degrees C					
	0% NaF		1% NaF		2% NaF		0% NaF		1% NaF		2% NaF		0% NaF		1% NaF		2% NaF	
Time	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE
0	0.45	0	0.45	0	0.47	0	0.45	0	0.45	0	0.47	0	0.45	0	0.45	0	0.45	0
7	0	0.014	0.32	NR	0.24	0.19	0	0.046	NR	NR	0.55	0.037	0.082	NR	0.49	0.078	0.45	0.021
7	0	0.025	0.21	0.14	0.21	0.14	0	0.052	0.47	0.035	0.46	0.041	0.083	0.028	0.48	0.019	0.47	0.116
7	0	0.060	0.24	0.15	0.26	0.15	0	0.029	0.45	0.056	0.45	0.051	0.087	0.033	0.46	0.040	0.34	0.051
Mean	0	0.033	0.26	0.14	0.24	0.16	0	0.042	0.46	0.046	0.49	0.043	0.084	0.030	0.48	0.045	0.42	0.062
14	0	0.014	0.015	0.28	0.008	0.30	0	0.018	0.22	0.19	0.28	0.15	NR	0.018	0.43	0.050	0.47	0.052
14	0	0.010	0.013	0.29	0.008	0.27	0	0.017	0.21	0.19	0.32	0.15	NR	0.016	0.47	0.051	0.46	0.052
14	0	0.012	NR	0.30	0.009	0.29	0	0.015	0.19	0.22	0.30	0.14	NR	0.015	0.43	0.051	0.44	0.066
Mean	0	0.012	0.014	0.29	0.008	0.29	0	0.017	0.21	0.198	0.30	0.15	NR	0.016	0.44	0.051	0.45	0.057
35	0	0.015	0	0.25	0	0.26	0	0.025	0.18	0.22	0.30	0.20	0.031	0.018	0.63	0.041	0.60	0.049
35	0	0.012	0	0.23	0	0.27	0	0.019	0.17	0.23	0.29	0.19	0.032	0.018	0.61	0.043	0.64	0.043
35	0	0.012	0	0.25	0	0.26	0	0.018	0.16	0.24	0.29	0.20	0.033	0.015	0.66	0.041	0.61	0.052
Mean	0	0.013	0	0.24	0	0.26	0	0.021	0.17	0.23	0.29	0.20	0.032	0.017	0.64	0.042	0.62	0.048
54	0	0.009	0	0.21	0	0.25	0	0.012	0.066	0.24	0.14	0.22	0.014	0.014	0.44	0.040	0.42	0.054
54	0	0.008	0	0.25	0	0.25	0	0.012	0.079	0.25	0.13	0.26	0.014	0.015	0.43	0.030	0.41	0.053
54	0	0.007	0	0.27	0	0.26	0	0.012	0.061	0.24	0.14	0.22	0.017	0.015	0.44	0.055	0.40	0.037
Mean	0	0.008	0	0.24	0	0.25	0	0.012	0.069	0.24	0.14	0.23	0.015	0.015	0.44	0.042	0.41	0.048
84	0	0	0	0.20	0	0.20	0	0	0.010	0.23	0.050	0.23	0	0	0.39	0.038	0.40	0.043
84	0	0	0	0.19	0	0.19	0	0	NR	0.25	0.051	0.24	0	0	0.38	0.040	0.43	0.040
84	0	0	0	0.21	0	0.21	0	0	0.007	0.27	0.062	0.23	0	0	0.43	0.041	0.40	0.039
Mean	0	0	0	0.20	0	0.20	0	0	0.008	0.25	0.054	0.23	0	0	0.40	0.040	0.41	0.041

Mean of triplicate GC-MS analyses given (n = 3 results per condition per analysis time). The mean of three extractions is given for Day 0. NR: no result

Appendix 22 Stability Study: Cocaine (COC) and benzoylecgonine (BZE) concentration (mg/L) measured in triplicate vitreous humour samples over 84 days

	20 degrees C				4 degrees C				-18 degrees C			
	0% NaF		1.5% NaF		0% NaF		1.5% NaF		0% NaF		1.5% NaF	
Time	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE
0	0.355	0	0.374	0	0.36	0	0.374	0	0.355	0	0.374	0
7	0.038	0.20	0.02	0.21	NR	NR	NR	NR	NR	NR	NR	NR
7	0.033	0.18	0.015	0.21	NR	NR	NR	NR	NR	NR	NR	NR
7	0.066	0.18	0.037	0.20	NR	NR	NR	NR	NR	NR	NR	NR
Mean	0.046	0.18	0.024	0.21	NR	NR	NR	NR	NR	NR	NR	NR
14	0.005	0.29	0.004	0.30	0.10	0.21	0.16	0.17	0.31	0.056	0.33	0.021
14	0.006	0.29	0.004	0.29	0.01	0.27	0.18	0.17	0.29	0.072	0.32	0.018
14	0.003	0.27	0.002	0.29	0.14	0.19	0.16	0.17	0.31	0.048	0.33	0.018
Mean	0.005	0.28	0.003	0.29	0.085	0.22	0.17	0.17	0.31	0.059	0.33	0.019
21	0	0.26	0	0.25	0.042	0.23	0.092	0.19	0.23	0.086	0.34	0.042
21	0	0.24	0	0.24	0.075	0.21	0.086	0.22	0.29	0.059	0.34	0.041
21	0	0.11	0	0.23	0.068	0.23	0.023	0.26	0.30	0.051	0.31	0.042
Mean	0	0.20	0	0.24	0.062	0.22	0.067	0.22	0.27	0.065	0.33	0.042
35	0	NR	0	0.25	0	0.30	0.049	0.25	0.33	0.03	0.33	0.017
35	0	0.18	0	0.25	0	0.25	0.041	0.25	0.34	0.019	0.35	0.012
35	0	NR	0	0.23	0	0.25	0.046	0.26	0.31	0.022	0.36	0.016
Mean	0	0.18	0	0.24	0	0.26	0.045	0.26	0.32	0.024	0.35	0.015
54	0	0.29	0	0.25	0	0.22	0.005	0.27	0.29	0.044	0.31	0.014
54	0	0.30	0	0.20	0	0.30	0.009	0.30	0.32	0.032	0.29	0.015
54	0	0.25	0	0.20	0	0.19	0.008	0.25	0.31	0.032	0.34	0.016
Mean	0	0.28	0	0.22	0	0.24	0.007	0.27	0.31	0.036	0.31	0.015
84	0	0.20	0	0.15	0	0.24	0.02	0.24	0.26	0.082	0.34	0
84	0	NR	0	0.14	0	0.2	0	0.24	0.21	0.094	0.32	0
84	0	0.20	0	0.15	0	0.21	0.01	0.23	0.30	0	0.31	0
Mean	0	0.20	0	0.15	0	0.22	0.010	0.24	0.26	0.059	0.32	0

Mean of duplicate GC-MS analyses given (n = 3 results per condition per analysis time). The mean of three extractions is given for Day 0. NR: no result

Appendix 23 Stability Study: Cocaine (COC) and benzoylecgonine (BZE) concentration (mg/L) measured in quadruplet muscle tissue samples over 31 days

	20 degrees C						4 degrees C						-18 degrees C					
	0% NaF		2% NaF		2% NaF/PBS		0% NaF		2% NaF		2% NaF/PBS		0% NaF		2% NaF		2% NaF/PBS	
Time	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE	COC	BZE
0	0.40	0	0.40	0	0.40	0	0.40	0	0.40	0	0.40	0	0.40	0	0.40	0	0.40	0
7	0.38	0.015	0.43	0	0.34	0.074	0.45	0	0.38	0	0.40	0	0.43	0	0.42	0	0.42	0
7	0.31	0.016	0.40	0	0.32	0.068	0.39	0	0.42	0	0.38	0	0.40	0	0.35	0	0.39	0
7	0.31	0.026	0.37	0	0.34	0.08	0.36	0	0.40	0	0.40	0	0.48	0	0.39	0	0.41	0
7	0.31	0.016	0.32	0	0.40	NR	NR	0	0.41	0	NR	0	0.31	0	0.41	0	0.41	0
Mean	0.33	0.018	0.38	0	0.35	0.074	0.40	0	0.40	0	0.39	0	0.41	0	0.39	0	0.41	0
14	0.32	0.076	0.38	0.036	0.25	0.14	0.40	0	0.40	0.002	0.41	0.015	0.39	0	0.43	0	0.41	0.003
14	0.32	0.075	0.37	0.043	0.24	0.12	0.40	0.003	0.42	0	0.39	0.018	0.39	0	0.42	0	0.43	0.002
14	0.30	0.090	0.38	0.036	0.25	0.15	0.40	0	0.42	0	0.41	0.012	0.41	0	0.43	0	0.41	0.010
14	0.29	0.069	0.36	0.037	0.26	0.15	0.40	0	0.42	0.003	0.41	0.000	0.41	0	0.42	0	0.41	0.003
Mean	0.31	0.078	0.37	0.038	0.25	0.14	0.40	0.001	0.42	0.001	0.40	0.011	0.40	0	0.43	0	0.42	0.005
31	0.097	0.31	0.34	0	0.13	0.26	0.4	0	0.43	0	0.38	0.017	0.48	0	0.41	0	0.43	0
31	0.075	0.31	0.33	0.11	0.12	0.28	0.34	0.081	0.43	0	0.39	0.024	0.44	0	0.45	0	0.44	0
31	0.094	0.32	0.33	0.094	0.14	0.24	0.43	0	0.46	0	0.42	0.02	0.45	0	0.43	0	0.42	0
31	0.086	0.30	0.33	0	0.14	0.27	0.40	0.051	0.46	0	0.44	0.018	0.45	0	0.46	0	0.46	0
Mean	0.088	0.31	0.33	0.051	0.13	0.26	0.39	0.033	0.45	0	0.41	0.02	0.46	0	0.44	0	0.44	0

The mean of three extractions is given for Day 0. NR: no result

Appendix 24 PH change in whole tissue, and homogenised tissue at 25, 4 and -18°C over 31 days storage

25 degrees C				
Time (days)	0	7	14	31
Whole tissue	5.7	6.2	6.1	7.0
Water homogenate	5.7	6.7	8.2	7.6
2% NaF homogenate	5.9	6.1	6.0	6.1
PBS/NaF homogenate	6.0	6.2	5.9	6.1

4 degrees C				
Time (days)	0	7	14	31
Whole tissue	5.7	5.8	5.8	5.8
Water homogenate	5.7	6.8	6.3	6.6
2% NaF homogenate	5.9	5.8	5.9	6.0
PBS/NaF homogenate	6.0	5.9	6.1	6.2

-18 degrees C				
Time (days)	0	7	14	31
Whole tissue	5.7	5.7	5.8	5.8
Water homogenate	5.7	5.8	5.8	5.9
2% NaF homogenate	5.9	6.1	6.0	6.2
PBS/NaF homogenate	6.0	6.3	5.9	6.1

Appendix 25 Stability Study: 6-Acetylmorphine (6AM) and morphine (MOR) concentration (mg/L) measured in triplicate blood samples over 84 days

	20 degrees C						4 degrees C						-18 degrees C					
	0% NaF		1% NaF		2% NaF		0% NaF		1% NaF		2% NaF		0% NaF		1% NaF		2% NaF	
Day	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR
0	0.14	0.034	0.15	0.032	0.15	0.028	0.14	0.034	0.15	0.032	0.15	0.028	0.14	0.034	0.15	0.032	0.15	0.028
7	0.015	0.10	0.11	0.058	0.12	0.061	0.066	0.083	NR	NR	0.13	0.037	0.13	0.052	0.15	0.052	0.13	0.047
7	0.018	0.097	0.098	0.057	0.11	0.055	0.065	0.079	0.13	0.068	0.12	0.037	0.12	0.056	0.14	0.050	0.14	0.060
7	0.016	0.095	0.11	0.056	0.12	0.060	0.066	0.080	0.13	0.063	0.13	0.037	0.13	0.050	0.14	0.045	0.099	0.039
Mean	0.016	0.098	0.10	0.057	0.12	0.058	0.066	0.081	0.13	0.066	0.13	0.037	0.13	0.053	0.14	0.049	0.12	0.049
14	0	0.091	0.064	0.10	NR	0.095	0.012	0.10	0.13	0.086	0.14	0.085	0.086	0.088	0.17	0.071	0.16	0.091
14	0	0.10	0.064	0.10	0.077	0.093	0.012	0.12	0.13	0.084	0.16	0.090	0.098	0.089	0.17	0.079	0.17	0.083
14	0	0.11	0.059	0.098	0.076	0.091	0.011	0.11	0.13	0.080	0.16	0.082	0.095	0.081	0.17	0.078	0.17	0.085
Mean	0	0.10	0.062	0.10	0.076	0.093	0.012	0.11	0.13	0.083	0.15	0.086	0.093	0.086	0.17	0.076	0.17	0.087
35	0	0.10	0.025	0.11	0.040	0.11	0	0.11	0.095	0.092	0.12	0.081	0.097	0.072	0.17	0.066	0.17	0.062
35	0	0.091	0.023	0.11	0.038	0.11	0	0.11	0.084	0.083	0.13	0.081	0.097	0.067	0.16	0.062	0.17	0.061
35	0	0.12	0.026	0.12	0.042	0.11	0	0.11	0.097	0.086	0.13	0.074	0.094	0.070	0.16	0.066	0.16	0.061
Mean	0	0.10	0.025	0.11	0.040	0.11	0	0.11	0.092	0.087	0.13	0.078	0.096	0.070	0.16	0.065	0.16	0.061
54	0	0.085	NR	0.10	0.021	0.11	0	0.089	0.066	0.086	0.099	0.078	0.072	0.082	0.14	0.057	0.15	0.064
54	0	0.10	0.014	0.11	0.022	0.11	0	0.094	0.073	0.082	0.089	0.080	0.081	0.076	0.15	0.056	0.14	0.062
54	0	0.086	0.014	0.11	0.022	0.10	0	0.096	0.068	0.092	0.10	0.072	0.084	0.077	0.15	0.060	0.15	0.060
Mean	0	0.092	0.014	0.11	0.021	0.11	0	0.093	0.069	0.087	0.096	0.077	0.079	0.078	0.15	0.058	0.15	0.062
84	0	0.091	0.004	0.11	0.008	0.11	0	0.082	0.044	0.093	0.079	0.080	0.075	0.072	0.14	0.054	0.14	0.059
84	0	0.083	0.004	0.11	0.007	0.11	0	0.088	0.046	0.095	0.079	0.081	0.080	0.063	0.14	0.054	0.14	0.057
84	0	0.084	0.004	0.11	0.007	0.11	0	0.089	0.043	0.099	0.083	0.080	0.080	0.060	0.14	0.056	0.14	0.058
Mean	0	0.086	0.004	0.11	0.007	0.11	0	0.086	0.044	0.096	0.080	0.080	0.078	0.065	0.14	0.055	0.14	0.058

Mean of triplicate GC-MS analyses given (n = 3 results per condition per analysis time). The mean of three extractions is given for Day 0. NR: no result

Appendix 26 Stability Study: 6-Acetylmorphine (6AM) and morphine (MOR) concentration (mg/L) measured in triplicate vitreous humour samples over 84 days

	20 degrees C				4 degrees C				-18 degrees C			
	0% NaF		1.5% NaF		0% NaF		1.5% NaF		0% NaF		1.5% NaF	
Time	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR
0	0.13	0.028	0.14	0.028	0.13	0.028	0.14	0.028	0.132	0.028	0.135	0.028
7	0.042	0.073	0.12	0.055	NA	NA	NA	NA	NA	NA	NA	NA
7	0.061	0.048	0.092	0.055	NA	NA	NA	NA	NA	NA	NA	NA
7	NR	0.092	0.11	0.053	NA	NA	NA	NA	NA	NA	NA	NA
Mean	0.052	0.071	0.11	0.054	NA	NA	NA	NA	NA	NA	NA	NA
14	0.033	0.09	0.093	0.057	0.07	0.073	0.13	0.045	0.081	0.064	0.13	0.033
14	0.036	0.08	0.092	0.058	0.046	0.078	0.14	0.042	0.076	0.065	0.13	0.032
14	0.009	0.092	0.095	0.063	0.076	0.071	0.14	0.041	0.074	0.059	0.14	0.028
Mean	0.026	0.087	0.093	0.059	0.064	0.074	0.14	0.043	0.077	0.063	0.14	0.031
21	0.012	0.092	0.054	0.094	0.021	0.089	0.13	0.086	0.061	0.091	0.13	0.07
21	0.014	0.093	0.057	0.089	0.062	0.089	0.13	0.085	NR	0.091	0.13	0.071
21	0	0.095	0.082	0.098	0.075	0.09	0.13	0.087	NR	0.088	NR	0.067
Mean	0.009	0.093	0.064	0.094	0.053	0.089	0.13	0.086	0.061	0.090	0.13	0.069
35	0	0.10	0.03	0.099	0.014	0.11	0.14	0.065	0.048	0.089	0.12	0.05
35	0	0.11	0.034	0.099	0	0.11	0.12	0.066	0.077	0.071	0.13	0.038
35	0	0.11	0.026	0.101	0.027	0.081	0.13	0.054	0.08	0.096	0.12	0.096
Mean	0	0.11	0.030	0.100	0.014	0.099	0.13	0.062	0.068	0.085	0.12	0.061
54	0	0.10	0.043	0.089	0	0.11	0.10	0.06	0.05	0.094	0.14	0.061
54	0	0.099	0	0.11	0.026	0.12	0.12	0.064	0.053	0.089	0.13	0.063
54	0	0.11	0	0.12	0	0.13	0.11	0.071	0.023	0.111	0.12	0.06
Mean	0	0.10	0.014	0.11	0.009	0.12	0.11	0.065	0.042	0.098	0.13	0.061
84	0	0.098	0.004	0.096	0	0.11	0.10	0.061	0.008	0.096	0.13	0.052
84	0	0.091	0.003	0.095	0	0.098	0.09	0.07	0	0.088	0.14	0.055
84	0	0.092	0.006	0.092	0	0.091	0.10	0.063	0.009	0.084	0.11	0.062
Mean	0	0.094	0.004	0.094	0	0.099	0.099	0.065	0.006	0.089	0.12	0.056

Mean of duplicate GC-MS analyses given (n = 3 results per condition per analysis time). The mean of three extractions is given for Day 0. NA: not analysed; NR: no result

Appendix 27 Stability Study: 6-Acetylmorphine (6AM) and morphine (MOR) concentration (mg/L) measured in quadruplet muscle tissue samples over 31 days

	20 degrees C						4 degrees C						-18 degrees C					
	0% NaF		2% NaF		2% NaF/PBS		0% NaF		2% NaF		2% NaF/PBS		0% NaF		2% NaF		2% NaF/PBS	
Time	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR	6AM	MOR
0	0.19	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03
7	0.16	0.055	0.17	0.048	0.17	0.055	0.20	0.035	0.19	0.042	0.19	0.037	0.17	0.039	0.18	0.035	0.20	0.037
7	0.16	0.048	0.19	0.046	0.17	0.048	0.20	0.04	0.19	0.039	0.19	0.044	0.17	0.026	0.17	0.041	0.15	0.033
7	0.15	0.047	0.18	0.05	0.17	0.050	0.17	0.03	0.18	0.032	0.20	0.039	0.17	0.026	0.18	0.043	0.19	0.036
7	0.16	0.044	0.17	0.043	0.19	0.045	0.13	0.027	0.18	0.035	NR	0.041	0.16	0.031	0.18	0.042	0.19	0.032
Mean	0.16	0.049	0.18	0.047	0.17	0.05	0.17	0.033	0.19	0.037	0.193	0.04	0.17	0.031	0.18	0.04	0.18	0.035
14	0.078	0.086	0.15	0.073	0.15	0.06	0.19	0.077	0.16	0.057	0.16	0.036	0.18	0.03	0.16	0.069	0.17	0.035
14	0.097	0.082	0.15	0.094	0.14	0.067	0.15	0.07	0.17	0.057	0.15	0.041	0.18	0.03	0.18	0.067	0.19	0.034
14	0.087	0.093	0.16	0.096	0.15	0.062	0.17	0.082	0.17	0.076	0.17	0.047	0.19	0.033	0.18	0.082	0.20	0.033
14	0.077	0.076	0.16	0.069	0.12	0.058	0.17	0.079	0.16	0.057	0.21	0.04	0.18	0.028	0.15	0.078	0.20	0.035
Mean	0.085	0.084	0.15	0.083	0.14	0.062	0.17	0.077	0.16	0.062	0.17	0.041	0.18	0.03	0.17	0.074	0.19	0.034
31	0	0.13	0.16	0.083	0.11	0.064	0.16	0.059	0.15	0.066	0.15	0.057	0.18	0.077	0.17	0.076	0.15	0.06
31	0	0.13	0.12	0.097	0.11	0.068	0.067	0.11	0.15	0.071	0.14	0.058	0.15	0.076	0.15	0.086	0.16	0.056
31	0	0.14	0.11	0.084	0.11	0.06	0.22	0.045	0.16	0.078	0.16	0.052	0.15	0.06	0.15	0.071	0.17	0.059
31	0	0.12	0.13	0.092	0.099	0.059	0.10	0.091	0.18	0.091	0.16	NR	0.19	0.068	0.16	0.071	0.17	0.059
Mean	0	0.13	0.13	0.089	0.11	0.063	0.14	0.077	0.16	0.077	0.15	0.056	0.17	0.07	0.16	0.076	0.16	0.059

The mean of three extractions is given for Day 0. NR: no result or flier (outlier)

Appendix 28 Concentrations of cocaine (COC), benzoylecgonine (BZE) and cocaethylene (COET) (mg/kg) measured in thigh muscle segments

Case no.	1			2			3			4		5	6			7		
ID	COC	BZE	COET	COC	BZE	COET	COC	BZE	COET	COC	BZE	BZE	COC	BZE	COET	COC	BZE	COET
A	0.048	0.88	0.050	0.046	0.40	0.11	0.38	1.3	0.009	0.024	0.10	0.32	0.21	0.77	0.019	0.47	0.16	ND
B	0.036	0.87	0.038	0.044	0.42	0.09	0.35	1.3	0.011	0.023	0.10	0.28	0.25	0.89	0.014	0.41	0.15	ND
C	0.033	0.87	0.032	0.037	0.41	0.081	0.34	1.4	ND	0.014	0.11	0.26	0.24	0.72	0.013	0.42	0.15	ND
D	0.021	0.89	0.025	0.049	0.37	0.10	0.35	1.5	0.002	ND	0.096	0.27	0.31	0.80	0.012	0.39	0.14	ND
E	0.023	0.85	0.027	0.051	0.36	0.11	0.34	1.3	0.008	0.014	0.075	0.30	0.28	0.85	0.014	0.35	0.14	0.002
F	0.022	0.85	0.029	0.051	0.47	0.11	0.34	1.3	0.007	ND	0.091	0.30	0.31	0.82	0.041	0.32	0.13	ND
G	0.027	0.88	0.032	NA	NA	NA	0.34	1.3	0.004	ND	0.093	0.29	0.30	0.83	0.016	0.36	0.14	ND
H	0.032	0.82	0.030	ND	ND	ND	0.33	1.3	0.001	0.016	0.11	0.30	0.25	0.86	0.021	0.37	0.14	ND
I	NR	0.84	0.031	0.035	0.39	0.077	0.37	1.3	0.005	ND	0.096	0.30	0.23	0.81	0.019	0.35	0.14	0.003
J	NR	0.88	0.039	0.050	0.40	0.096	0.39	1.3	0.003	ND	0.090	0.29	0.25	0.85	0.017	0.37	0.15	0.003
K	NR	1.1	0.025	0.044	0.35	0.095	0.35	1.3	0.002	ND	0.10	0.30	0.28	0.85	0.022	0.37	0.14	0.002
L	NR	0.90	0.035	0.043	0.36	0.11	0.35	1.4	0.002	ND	0.094	0.28	0.33	0.88	0.015	0.42	0.15	ND
Mean	0.029	0.88	0.032	0.046	0.39	0.097	0.35	0.14	0.005	0.018	0.098	0.29	0.27	0.84	0.018	0.38	0.14	0.003

COET not detected (ND) in case 4 and 5; cocaine not detected in case 5.

NR: no result

Appendix 29 Concentrations of cocaine (COC), benzoylecgonine (BZE) and cocaethylene (COET) measured in sub-sections of thigh muscle

Case no.	Sample ID	COC (mg/kg)	BZE (mg/kg)	COET (mg/kg)
1	F.1	0.021	0.82	0.028
	F.2	0.023	0.88	0.031
	K.1	NR	0.89	0.021
	K.2	NR	0.90	0.029
	Mean	0.022	0.87	0.027
2	I.1	0.048	0.39	0.098
	I.2	ND	ND	ND
	I.3	0.048	0.40	0.098
	I.4	0.053	0.42	0.094
	Mean	0.050	0.40	0.097
3	D.1	0.30	1.4	< 0.01
	D.2	0.40	1.4	< 0.01
	D.3	0.40	1.7	< 0.01
	D.4	0.32	1.4	< 0.01
	Mean	0.35	1.5	
4	K.1	ND	0.099	ND
	K.2	ND	0.089	ND
	K.3	ND	0.10	ND
	K.4	ND	0.11	ND
	Mean		0.10	
5	E.1	ND	0.30	ND
	E.2	ND	0.29	ND
	E.3	ND	0.30	ND
	E.4	ND	0.30	ND
	Mean		0.298	
6	D.1	0.27	0.91	0.016
	D.2	0.27	0.84	0.014
	D.3	0.27	0.89	0.013
	D.4	0.29	0.77	0.014
	Mean	0.28	0.86	0.014
7	B.1	0.41	0.15	ND
	B.2	0.42	0.14	ND
	H.1	0.39	0.14	ND
	H.2	0.35	0.15	ND
	Mean	0.38	0.15	

NR: no result; ND: not detected

Segments locations are displayed in Figure 18

Appendix 30 Tissue distribution of cocaine, BZE and COET in 9 cases of cocaine involved death

Concentration in all matrices (mg/L; mg/kg)						
Case no.	Compound	CB	FB	VH	ThM	CM
1	Cocaine	0.016	0.015	0.26	0.029	0.034
	BZE	1.1	0.66	0.82	0.88	0.94
	COET	0.014	0.019	0.071	0.032	0.063
2	Cocaine	0.013	0.020	0.27	0.046	0.059
	BZE	0.22	0.25	0.33	0.39	0.42
	COET	0.055	0.052	0.18	0.097	0.21
3	Cocaine	0.032	0.089	0.53	0.35	0.73
	BZE	0.77	0.88	0.82	1.3	1.5
	COET	< 0.01	0	0	<0.01	0.011
4	Cocaine	0	<0.01	0.053	0.018	0
	BZE	0.078	0.030	0.082	0.097	0.1
	COET	0	0	0	0	0
5	Cocaine	0.016	NS	0.33	0	0
	BZE	0.42	NS	0.41	0.29	0.25
	COET	0	NS	0	0	0
6	Cocaine	0.11	NS	0.31	0.27	0.32
	BZE	0.71	NS	0.96	0.84	0.80
	COET	<0.01	NS	0.061	0.018	0.019
7	Cocaine	0.16	0.17	0.46	0.38	0.43
	BZE	0.19	0.17	0.21	0.14	0.15
	COET	<0.01	<0.01	0.021	<0.01	0
8	Cocaine	0	NS	<0.01	0	0
	BZE	0	NS	0.011	0	0
	COET	0	NS	0	0	0
9	Cocaine	<0.01	0	0.013	0	0
	BZE	0.017	0	0.054	0	0
	COET	<0.01	0	<0.01	0	0

CB: cardiac blood; FB: femoral blood; VH: vitreous humour; ThM: thigh muscle; CM: cardiac muscle

NS = no sample available for analysis

Appendix 31 Concentrations of cocaine (COC), benzoylecgonine (BZE) and cocaethylene (COET) measured in left and right femoral blood (FB) and vitreous humour (VH) samples

Case no.	Side	COC (mg/L)		BZE (mg/L)		COET (mg/L)	
		FB	VH	FB	VH	FB	VH
1	Right	0.010	0.25	0.63	0.82	0.014	0.070
	Left	0.020	0.27	0.69	0.82	0.024	0.071
	Mean	0.015	0.26	0.66	0.82	0.019	0.071
	% Deviation	33	5	4	0	26	0
2	Right	0.016	0.26	0.23	0.30	0.039	0.17
	Left	0.023	0.29	0.27	0.36	0.064	0.18
	Mean	0.020	0.27	0.25	0.33	0.052	0.18
	% Deviation	19	5	8	10	25	3
3	Right	0.077	0.71	0.81	1.0	0	0
	Left	0.100	0.35	0.94	0.65	0	0
	Mean	0.089	0.53	0.88	0.82		
	% Deviation	13	34	7	21		
4	Right	BLOQ	0.063	0.035	0.080	0	0
	Left	BLOQ	0.044	0.025	0.083	0	0
	Mean		0.053	0.030	0.082		
	% Deviation		17	-17	2		
5	Right	NS	0.33	NS	0.41	NS	0
	Left	NS	NS	NS	NS	NS	0
	Mean		0.33		0.41		
	% Deviation		-		-		
6	Right	NS	0.28	NS	0.95	NS	0.055
	Left	NS	0.34	NS	0.97	NS	0.066
	Mean		0.31		0.96		0.061
	% Deviation		9		1		9
7	Right	0.15	0.49	0.17	0.20	0	0.022
	Left	0.18	0.43	0.17	0.22	0.003	0.019
	Mean	0.17	0.46	0.17	0.21	0.003	0.021
	% Deviation	9	7	0	5	100	7
8	Right	NS	BLOQ	NS	0.010	NS	0
	Left	NS	BLOQ	NS	0.011	NS	0
	Mean				0.011		
	% Deviation						
9	Right	NS	0.013	NS	0.052	NS	0.004
	Left	0	0.012	0	0.056	0	0
	Mean		0.013		0.054		0.004
	% Deviation		4		4		100

Appendix 32 Comparison of opiate results obtained in initial test (carried out at the Department of Forensic Medicine and Pathology, Dundee) and at re-test (present analysis carried out at Bournemouth University)

Case	Time stored (months)	Free morphine in femoral blood (mg/L)					6AM detection (matrix)	
		Initial result	Re-test (pooled)	Mean	Difference (re-test – initial)	Deviation (%)	Initial	Re-test
1	5	0.19	0.29	0.24	-0.098	21	U	VH+FB
2	6	0.3	0.20	0.25	0.102	-20	U	VH+FB
3	6	0.13	0.15	0.14	-0.022	8	‡	VH+FB
4	7	0.32	0.40	0.36	-0.075	10	U	VH+FB
5	8	0.34	0.38	0.36	-0.037	5		VH+FB
6	9	0.09	0.095	0.09	-0.005	3		
7	8	0.09	0.12	0.10	-0.025	12		
8	9	0.017	0.018	0.02	-0.001	3	U	
9	9	0.06*	0.024	0.04	0.036	-43	U	VH
10	10	0.4	0.30	0.35	0.103	-15		VH+FB
11	10	0.3	0.24	0.27	0.061	-11		VH+FB
12	11	0.15	0.14	0.14	0.012	-4	U	VH
13	11	0.08	0.083	0.08	-0.003	2		FB
14	11	0.24	0.22	0.23	0.025	-5		VH
15	7	0.25	0.15	0.20	0.100	-25		VH
16	11	0.05	0.12	0.09	-0.070	41	U	VH
17	12	0.26	0.22	0.24	0.037	-8		VH
18	13	0.09	0.16	0.13	-0.070	28		VH
19	14	0.32	0.31	0.32	0.007	-1		VH
20	14	0.41	0.40	0.40	0.014	-2		VH+FB
21	30	0.39	0.083	0.24	0.307	-65		VH
22	?	0.28	0.18	0.23	0.099	-21	†	VH
23	29	0.2	0.13	0.16	0.071	-22		VH
24	28	1.2	0.83	1.02	0.370	-18		

Appendix 32 continued

Case	Time stored (months)	Free morphine in femoral blood (mg/L)					6AM detection (matrix)	
		Initial result	Re-test (pooled)	Mean	Difference (re-test – initial)	Deviation (%)	Initial	Re-test
25	27	0.15	0.087	0.12	0.063	-27		
26	27	0.18	0.064	0.12	0.116	-48		VH
27	27	0.11	0.12	0.11	-0.006	3		VH+FB
28	26	0.41	0.28	0.35	0.129	-19		VH
29	25	0.12	0.088	0.10	0.032	-15		VH
30	25	4.4	4.8	4.60	-0.400	4		
31	24	0.14	0.069	0.10	0.071	-34		
32	24	0.16	0.11	0.14	0.048	-18		
33	24	0.16	0.16	0.16	0.003	-1	‡	VH+FB
34	24	0.26*	0.18	0.22	0.084	-19		VH
35	24	0.17	0.22	0.20	-0.054	14		VH
36	23	0.22	0.20	0.21	0.016	-4		VH
37	22	0.12	0.11	0.12	0.010	-4		VH
38	22	0.12*	0.21	0.16	-0.089	27	‡	VH
39	22	0.024	0.008	0.02	0.016	-50		
40	21	0.029	0.009	0.02	0.020	-53		
41	20	0.13	0.16	0.15	-0.030	10		VH
42	19	0.41	0.47	0.44	-0.063	7		VH+FB
43	19	0.04	0.040	0.04	0.000	0		VH
44	18	0.09	0.024	0.06	0.066	-58	U	VH
45	18	0.077	0.12	0.10	-0.047	23	U	VH+FB
46	18	0.26	0.27	0.26	-0.008	2	U	VH+FB
47	18	0.25	0.38	0.32	-0.132	21		VH+FB
48	17	0.22	0.33	0.27	-0.109	20	U	VH
49	17	1.3	0.89	1.10	0.407	-19	†	VH

Appendix 32 continued

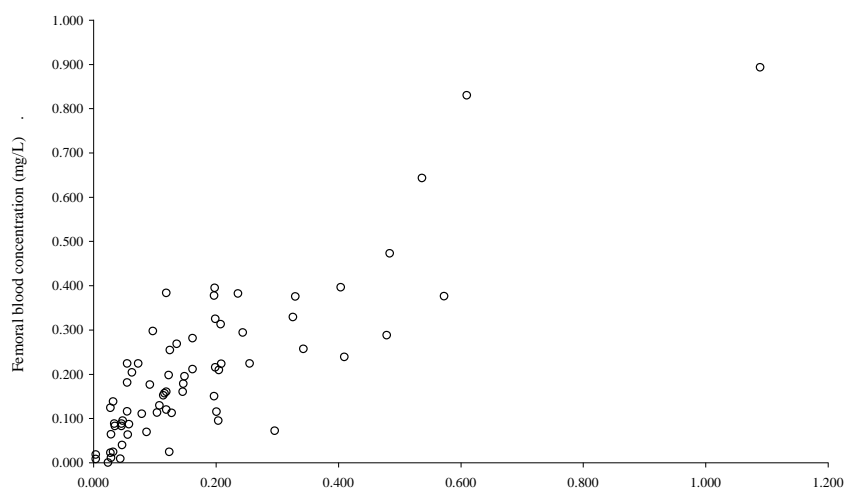
Case	Time stored (months)	Free morphine in femoral blood (mg/L)					6AM detection (matrix)	
		Initial result	Re-test (pooled)	Mean	Difference (re-test – initial)	Deviation (%)	Initial	Re-test
50	17	0.24	0.29	0.27	-0.054	10		VH
51	17	0.14	0.22	0.18	-0.084	23	U	VH
52	16	0.26	0.26	0.26	0.003	-1	U	
53	16	0.12	0.22	0.17	-0.104	30	U	VH
54	16	0.04	0.088	0.06	-0.048	38		VH
55	16	0.06	0.063	0.06	-0.003	2		VH
56	16	0.24	0.38	0.31	-0.136	22	U	VH+FB
57	12	0.15	0.095	0.12	0.055	-22	U	VH
58	14	0.05*	0.072	0.06	-0.022	18	U	
59	12	0.19	0.11	0.15	0.077	-25	U	VH
60	14	0.12	0.21	0.17	-0.091	27	U	VH
61	11	0.38**	0.18	0.28	0.202	-36	U	VH+FB
62	9	0.54	0.38	0.46	0.165	-18	U	VH+FB
63	8	0.29	0.25	0.27	0.036	-7	U	VH+FB
64	?	0.03	0.022	0.03	0.008	-15	U	VH
65	7	0.35	0.38	0.37	-0.033	5	U	VH+FB
66	6	0.28	0.33	0.30	-0.045	7	FB (†)	VH+FB
67	6	0.17	0.20	0.18	-0.025	7		VH
68	4	0.021	0.011	0.02	0.010	-31	FB+U	VH
69	3	0	0	0.00	0.000	0		
70	11	0.61	0.64	0.63	-0.033	3	U	VH+FB

* Subclavian blood result; ** Cardiac blood result;

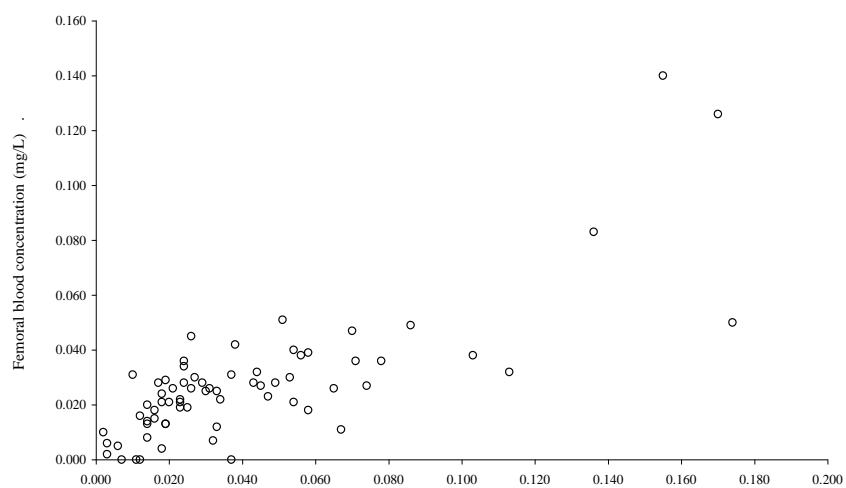
U: Urine; FB: femoral blood; VH: vitreous humour

† No urine sample available; ‡ availability of urine sample unknown

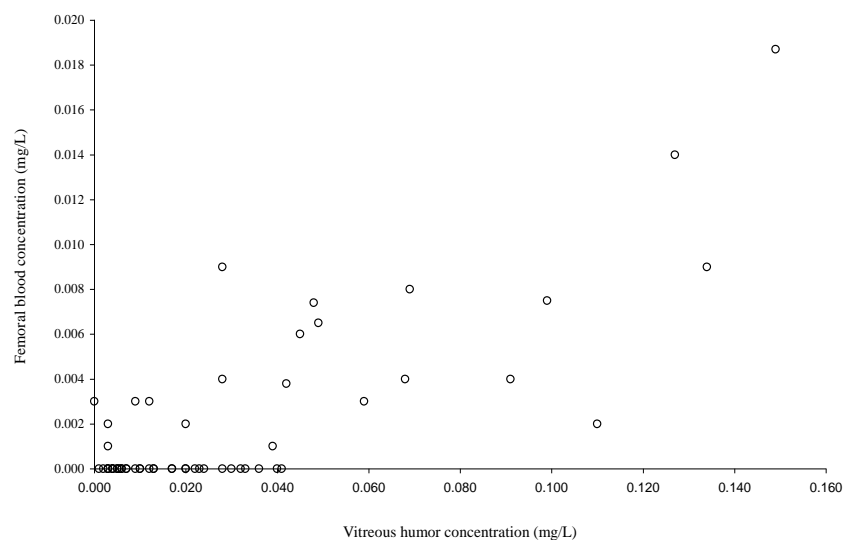
Appendix 33 Relationship between femoral blood and vitreous humour concentration of (A) free morphine (excluding case 30; n = 69), (B) codeine (excluding case 69; n = 69) and (C) 6AM (n =57)



(A)

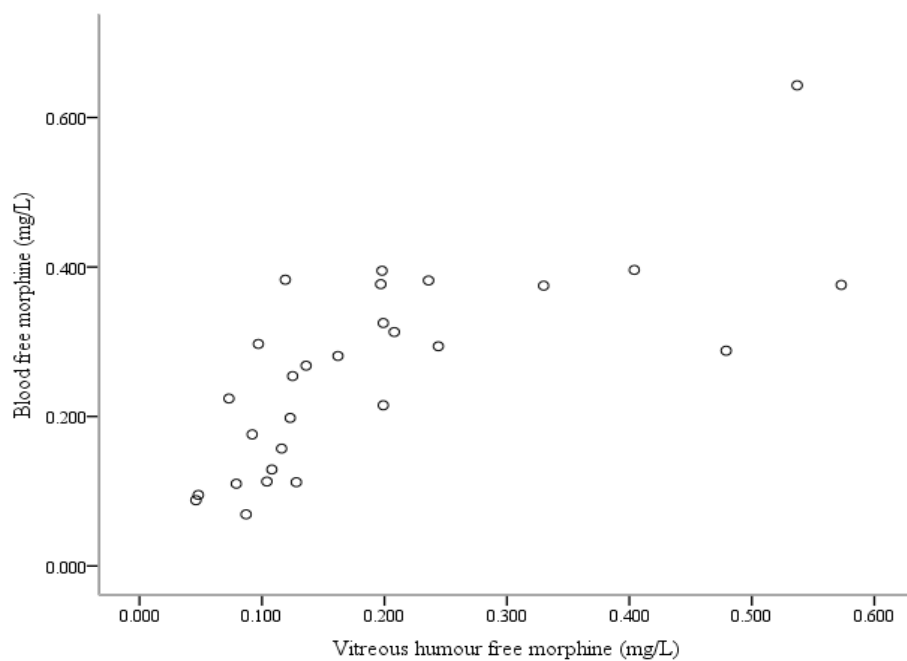


(B)

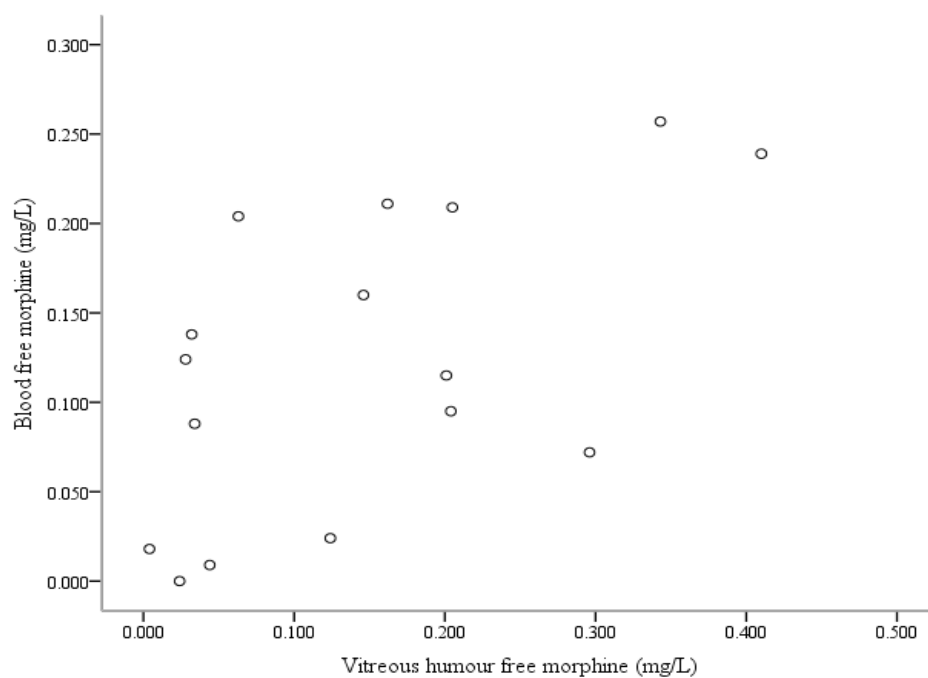


(C)

Appendix 34 Correlation between femoral blood and vitreous humour free morphine concentration (mg/L) in (A) rapid deaths (n = 28) and (B) delayed deaths (n = 16)

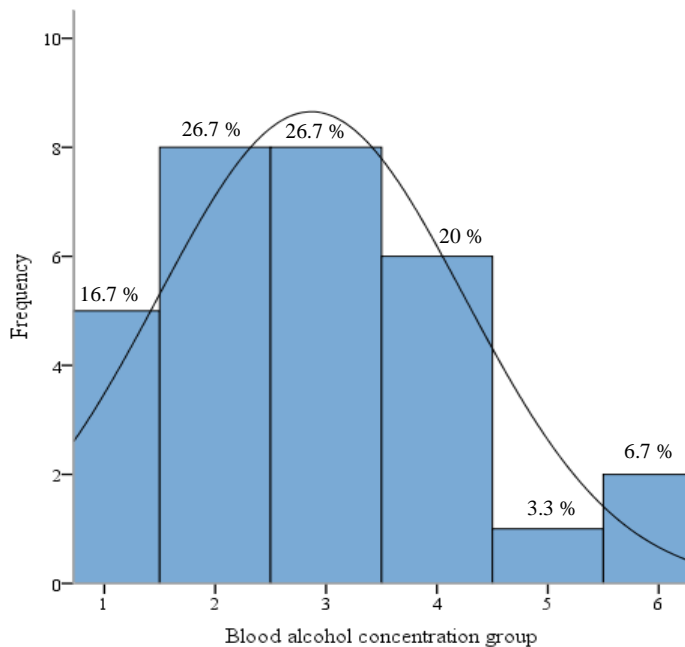


(A)

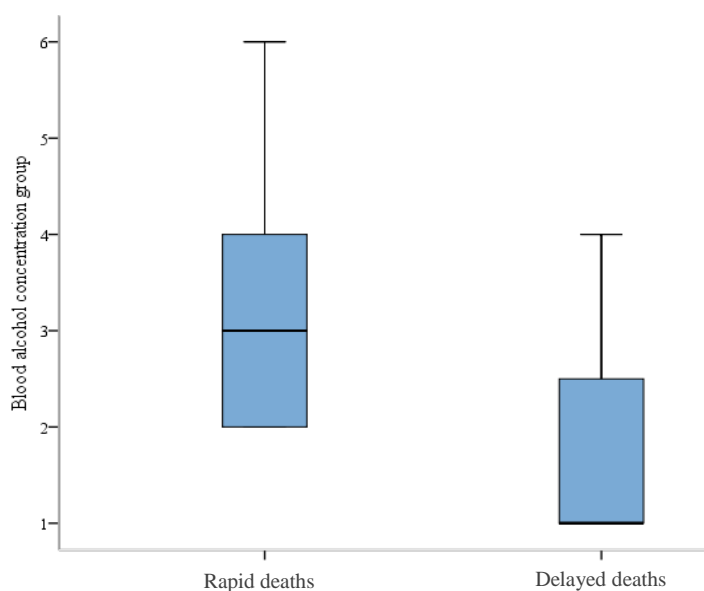


(B)

Appendix 35 Distribution frequency of blood alcohol concentration measured in (A) alcohol positive morphine involved deaths (n = 30) and in (B) rapid (n = 14) and delayed (n = 4) deaths (with standard error shown on box plot)



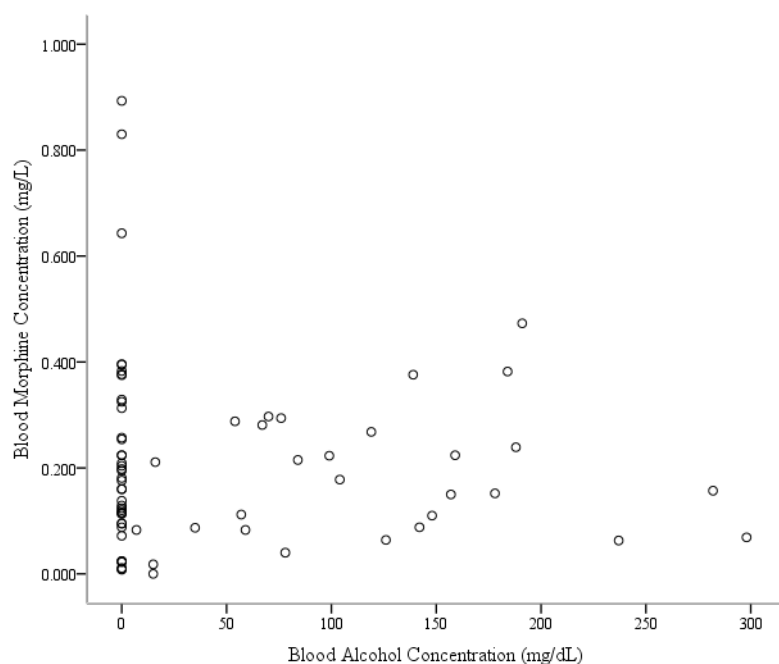
(A)



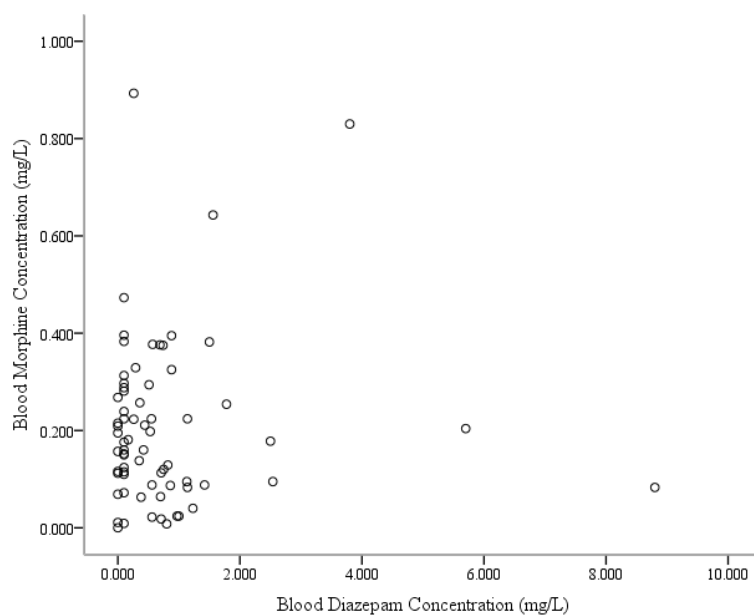
(B)

Concentration group (mg/dL): [1] < 50; [2] 50 – 100; [3] 100 – 150 [4] 150 – 200; [5] 200 – 250 [6] > 250

Appendix 36 Correlation between femoral blood morphine concentration and (A) alcohol concentration and (B) diazepam concentration in 70 morphine-related deaths



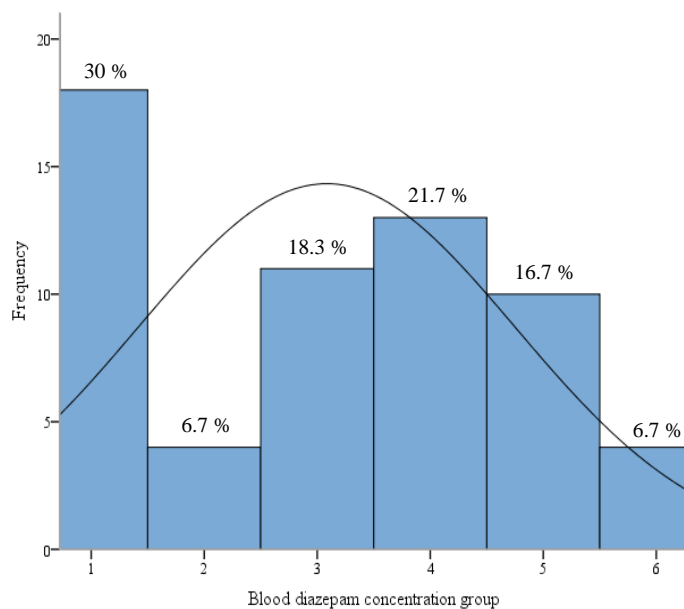
(A) $N = 69$; $r = -.053$; $p > .05$



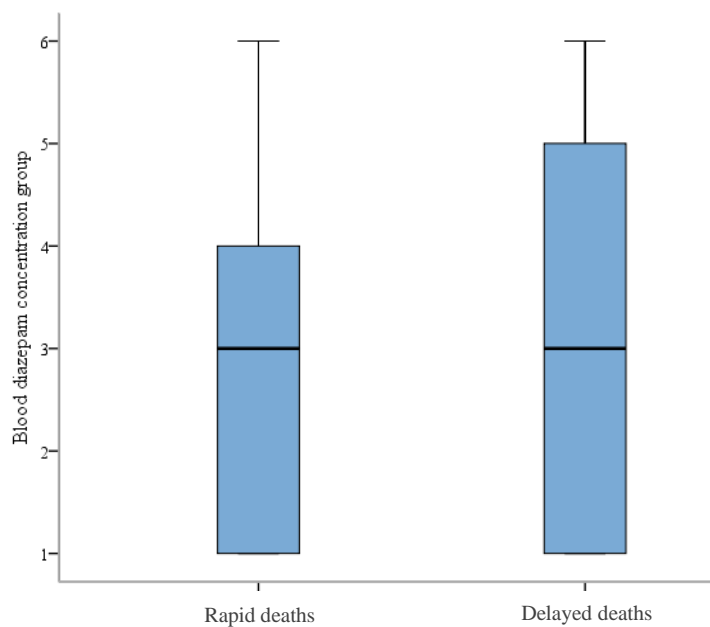
(B) $N = 69$; $r = .060$; $p > .05$

Spearman's rho correlation coefficients (r_s) and significance levels (p) are reported

Appendix 37 Distribution frequency of blood diazepam concentration measured in (A) morphine involved deaths (n = 60) and in (B) rapid (n = 28) and delayed (n = 16) deaths (with standard error shown on box plot)



(A)



(B)

Concentration group (mg/L): [1] < 0.16; [2] 0.16 – 0.3; [3] 0.3 – 0.6 [4] 0.6 – 0.9; [5] 0.9 – 1.6 [6] > 1.6